A DISSERTATION ON

A SHORT REPORT ON INHIBITORY POTENTIAL OF CARVACROL AGAINST 3-HYDROXY-3-METHYLGLUTARYL COENZYME-A REDUCTASES: AN IN-SILICO AND IN-VITRO STUDY

SUBMITTED TO THE

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BY

KEHKASHAN REHMAN
M.Sc. (Hons.) Biotechnology (IV Semester)
Department of Biosciences
INTEGRAL UNIVERSITY, LUCKNOW

UNDER THE SUPERVISION OF

Dr. M. SALMAN KHAN
Associate Professor
Department of Biosciences
Integral University, Lucknow

INTEGRAL UNIVERSITY

Established Under U.P. Act No 09 of 2004 by State Legislation
Approved by University Grants Commission
Phone No.: +91 (0552) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026, Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that **Ms. KEHKASHAN REHMAN**, a student of M.Sc. (Hons.) Biotechnology (II Year, IV semester), Integral University has completed her four months dissertation work entitled ***"A short report on inhibitory potential of carvacrol against 3-hydroxy-3-methylglutaryl coenzyme-a reductases: an in-silico and in-vitro study"*** successfully. She has completed this work from Department of Biosciences, Integral University, under the guidance of **Dr. M. Salman Khan**

The dissertation was a compulsory part of her M.Sc. degree. I wish her good luck for the future endeavours.

Dr. Snober S. Mir
Head,
Department of Biosciences,
Integral University, Lucknow

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TO WHOM IT MAY CONCERN

This is to certify that the study conducted by **Ms. KEHKASHAN REHMAN** during the months February–June, 2022 reported in the present thesis was under my guidance and supervision. The results reported by her are genuine and script of the thesis has been written by the candidate herself. The thesis entitled is ***"A short report on inhibitory potential of carvacrol against 3-hydroxy-3-methylglutaryl coenzyme-a reductases: an in-silico and in-vitro study"*** therefore, being forwarded for the acceptance in partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology, Department of Biosciences, Integral University, Lucknow.

Date:

Place: Lucknow

Dr. M. Salman Khan

(Supervisor)

Associate Professor
Department of Biosciences
Integral University, Lucknow

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Before I present my work, I would like to gratefully acknowledge the contribution of all those people who have helped in the work described in this Dissertation. I am going to try anyway, and if your name is not listed, it is rest assured that my gratitude is not less than for those listed below.

First, I would like to express my gratitude to **God** for providing me the blessing to complete this work.

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Date: **KEHKASHAN REHMAN**

CONTENTS

ABBREVIATIONS

DM	Diabetes Mellitus
T1DM	Type-1 Diabetes Mellitus
T2DM	Type-2 Diabetes Mellitus
IDDM	Insulin dependent diabetes mellitus
NIDDM	Non-insulin dependent diabetes mellitus
DPPH	2,2-diphenyl-1-picryl hydrazyl
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
EtOAc	Ethyl acetate
MeOH	Methanol
DCM	Dichloromethane
PBS	Phosphate buffered saline
DNS	Dinitrosalicylic acid
CVD	Cardiovascular disease
HbA1C	Glycated Heamoglobin

INTRODUCTION

Hypercholesterolemia and its induced oxidative stress are now considered to be one of the major contributors in progression of atherosclerosis. Cardiovascular disease (CVD) is the major cause of death in developed and developing countries (Guilbert, 2003). An excessive concentration of lipids in plasma may alter the lipoprotein metabolism and results in low density lipoprotein (LDL) accumulation in sub-endothelial space of arteries where it undergoes oxidative modifications to form oxidized LDL (Griendling, 2003) which is highly atherogenic. Several risk factors like hypercholesterolemia and cholesterol-induced oxidative stress enhance the formation of reactive oxygen species (ROS) which leads to the advancement of atherosclerotic lesions in vascular wall (Byon, 2008). Studies have reported that elevated lipid level, like total cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL) cholesterol, and a decrease in high density lipoprotein (HDL) cholesterol are directly associated with hyperlipidemia and atherosclerosis (Lee et al., 1980). It is well known that three major risk factors for CVD are hypercholesterolemia, smoking and hypertension (Stocker, 2004).

The cholesterol synthesis is regulated by β-hydroxy-β-methylglutaryl- CoA reductase (HMG-R) (EC 1.1.1.34), the rate limiting enzyme of cholesterol pathway (Brown and Goldstein 1980), and catalyses the conversion of HMG-CoA to mevalonic acid. Currently prescribed drugs that lower cholesterol level mainly work by inhibiting the HMG-CoA reductase enzymatic activity (Carbonell 2005). Drugs that lower cholesterol level mainly work by inhibiting the HMG-CoA reductase activity (Corsini *et al.,* 1995). Treatment and management of hyperlipidemia include dietary changes, weight loss, and use of hypolipidemic drugs. Currently, prescribed synthetic hypolipidemic agents (simvastatin, lovastatin, pravastatin, fluvastatin, etc.) that have been used for decades results in prevalence of their adverse side effects such as liver damage and myopathy (Golomb and Evans, 2008). In order to reduce the progression of CVDs in the body, different drugs such as statins are used frequently. Statins specifically inhibit the HMG-R, which is the rate limiting enzyme of the cholesterol synthesis (Pella *et al.,* 2005). Statins in combination with fibrates show significant benefit at higher doses but are also associated with severe side effects (Reyes-Soffer 2013).

Nonetheless, these oral medications have certain limitation and side effects (Golomb 2008). Long term use of statins also has host of side effects and may incurve problems in terms of toxicity and cost. Therefore, naturally derived therapeutic agents which are non-cytotoxic, free radical scavenger, and have the ability to lower cholesterol level in the system by inhibiting HMG-CoA reductase activity are in high demand for the treatment of hyperlipidemia and cholesterol-induced oxidative stress as well as atherosclerosis. Plant products are less toxic, have no side effects and free radical scavengers, and are now considered to be the best source for new hypolipidemic drugs and atherosclerosis development programme (Iqbal et al., 2013).

Number of studies has demonstrated the role of natural products in inhibiting the HMG-CoA reductase activity (Iqbal *et al.,* 2014a; Reddy et al., 2014; Iqbal *et al.,* 2014b) and hypercholesterolemia (Iqbal *et al.,* 2015) as well as atherosclerosis (Khan *et al.*, 2011). Plants, dietary fibres, and other food products are best source of new drugs and are widely used as cholesterol lowering agents and HMG-CoA reductase inhibitors (Khan *et al.,* 2011). A plant dominated diet mainly has multiple nutrients such as vitamins, carotenoids, and flavonoids that may influence the risk of CVD by preventing the oxidation of cholesterol in arteries. Carotenoids are mainly derived from entire plant such as roots, stems, leaves and flowers. Human blood and tissues have measurable concentrations of approximately 12 carotenoids that account for most of the dietary intake (Crews *et al.,* 2001). The most common of the carotenoids are: lycopene, lutein, α-carotene, β-carotene, β-cryptoxanthin and zeaxanthin (Stahl and Sies*,* 1995). Among these, lycopene received much attention over the world due to its potential antioxidant and anti-atherosclerotic properties.

Recently, involvement of inflammation in atherosclerosis has spurred the discovery and adoption of inflammatory biomarkers for cardiovascular risk prediction. Two hypothesises have been proposed to explain the role of inflammatory markers in the pathogenesis of atherosclerosis. One mechanism may be the ongoing inflammation in the artery, stimulated by the oxidized-low-density lipoprotein (ox-LDL), which leads to the production of cytokines that may induce the various acute phase proteins. Alternatively, chronic elevations of acute phase

reactants could be due to smoking, chronic infections, obesity and hypercholesterolemia, all of which contribute to the development of atherosclerosis. The second one is hyperlipidemia induced atherosclerosis. Among more than 600 carotenoids in plants, only about 14 are found in human tissues (Khachik *et al*., 1995). Tomato and tomato products contribute to nine of these 14 carotenoids and are the predominant source of lycopene, neurosporene, gamma-carotene, phytoene, and phytofluene. Because lycopene has potent antioxidant properties (Sies 1995).

Medicinal plants are the main source of numerous valuable phytochemicals with a wide range of application in human life. Many of these natural chemical compounds such as monoterpenes, flavanoids, isoflavones, saponins, alkaloids, etc. possess biological and pharmacological activities, which are intensively studied (Dorni et al., 2017). Monoterpenoids from essential oils of many medicinal herbs are used as flavor additives in food and beverages and as fragrance in cosmetics, household products and perfumes (Tchimene et al., 2013). Monoterpenoids are also an excellent alternative as antibacterial and antifungal agents. Numerous natural plant compounds and essential oils are also successfully used as antimutagens and antigenotoxins (Kumar et al., 2014, Eke and Çelik, 2016, Shruthi and Vijayalaxmi, 2016, Bacanlı et al., 2017, Ruiz et al., 2017).

The acyclic monoterpenoid geraniol is a common component of the essential oils of plant species such as citrus, lemon grass, ginger, lavender, geranium, nutmeg, palmarosa, mirtus and rose – Bulgarian *Rosa alba* L. and *Rosa damascena* Mill. (Baharvand-Ahmadi et al., 2015, Sharma et al., 2016). Geraniol is used in cosmetics, shampoos, toilet soaps etc. because of its fine fragrance (Burdock, 2016). This natural compound constitutes 18.28% of all the oil ingredients of R. alba L. essential oil. There are many studies on its biological activities. It has an inflamatory effect (Khan et al., 2013), and the inhalation of both geraniol and C. martinii essential oil can reduce the total cholesterol in rats (Andrade et al., 2014). Geraniol shows well expressed antimutagenic, antitumor and anticancer effects (Kim et al., 2012, Madankumar et al., 2013). Kim et al., reported that this phytochemical inhibited the growth of prostate cancer by targeting cell cycle and apoptosis pathways. Burke et al. (Burke et al., 2002)

investigated the mechanism of action of geraniol against pancreatic tumours. Shanmugapriya et al. (2017) obtained that the monoterpenoid could act as a potent antiproliferative agent against MNNG-induced endometrial cancer. Geraniol also shows good larvicide and insecticide activities (George et al., 2009; Qualls and Xue, 2009) and is an effective fumigant (Cornelius et al., 1997). Geraniol shows antifungal properties against pathogenic Candida albicans, protects against vaginal infection (Maruyama et al., 2008) and can inhibit the growth of some pathogens such as Salmonella typhimurium and *E. coli* (Si et al., 2006). In order to be used in human practice, natural plant compounds must be biosafe, noncytotoxic and nongenotoxic. Geraniol was nontoxic for rats using glucose, urea, creatinine, glutamic oxaloacetic transaminase (SGOT) and glutamic pyruvate transaminase (SGPT) as biochemical parameters (Farhath et al., 2012).

REVIEW OF LITERATURE

Stress

Today, the entire world is witnessing an upsurge in chronic health complications like cardiovascular disease, hypertension, diabetes mellitus, different forms of cancer, and other maladies. Medical surveys suggest that diet may serve as a potential tool for the control of these chronic diseases (Kumar et.al, 2011). The term “stress” has been used in physics since unknown time as it appears in the definition of Hooke’s law of 1658, but its first use in the biological science dates back to Sir Hans Selye’s letter to the Editor of Nature in1936. At that time, it was not accepted, but later on, after the famous address of Hans Selye at the prestigious College of France, it received approval among scientific community, but defining stress again troubled Selye over several years.

Today, stress can be defined as a process of altered biochemical homeostasis produced by psychological, physiological, or environmental stressors. Any stimulus, no matter whether social, physiological, or physical, that is perceived by the body as challenging, threatening, or demanding can be labelled as a stressor. The presence of a stressor leads to the activation of neurohormonal regulatory mechanisms of the body, through which it maintains the homeostasis (Dimitrios *et al*., 2003). The overall physiological impact of these factors and the adaptation ability of the body determine the variations in growth, development, productivity, and health status of the animals (K. Lundgren *et al*., 2013).

Oxidative Stress

The harmful effect of free ROS and RNS radicals causing potential biological damage is termed oxidative stress and nitrosative stress, respectively (Ridnour *et al.*, 2005). This is evident in biological systems when there is either an excessive production of ROS/RNS and/or a deficiency of enzymatic and non-enzymatic antioxidants. The redox stress/oxidative stress is a complex process. Its impact on the organism depends on the type of oxidant, on the site and intensity of its

production, on the composition and activities of various antioxidants, and on the ability of repair systems (Durackova *et al*., 2007).

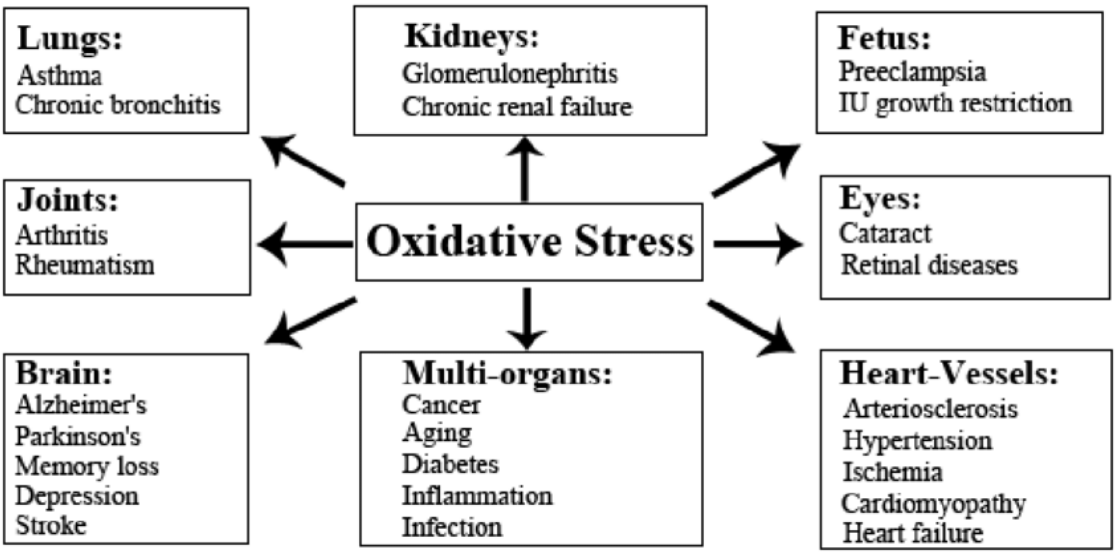


Figure 1: Oxidative stress and disease development.

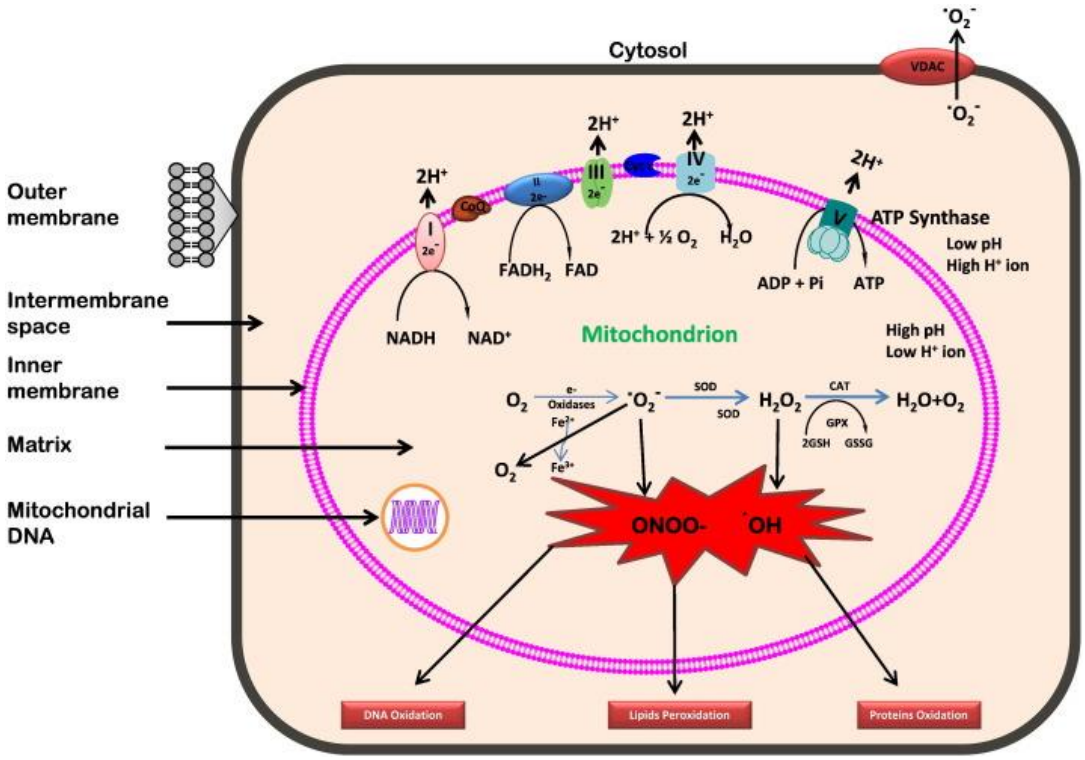


Figure 2: Oxidative stress and mitochondrial dysfunction

The close association between oxidative stress and lifestyle-related diseases has become well known. Oxidative stress is defined as a “state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them.” It not only causes hazardous events such as lipid peroxidation and

oxidative DNA damage, but also physiologic adaptation phenomena and regulation of intracellular signal transduction.

However, oxidative stress is actually useful in some instances. For example, oxidative stress induces apoptosis to prepare the birth canal for delivery. Also, biological defence mechanisms are strengthened by oxidative stress during appropriate physical exercise and ischemia. Therefore, a more useful definition of oxidative stress may be a "*state where oxidation exceeds the antioxidant systems because the balance between them has been lost*" (Toshikazu yoshikawa 2002). Today the world is experiencing a rise in age related chronic health diseases like cardiovascular disorders, cancer, and so forth and their associated negative health impacts and mortality/casualty (Ballinger 2005). Some metabolic diseases like diabetes are also associated with an enhanced level of lipoperoxidation.

Free radicals

The father of free radical research, Dr. Denham Harman, proposed his free radical theories in 1956. Free radicals are atoms or molecules containing an odd number of electrons, which results in an odd electron in the external orbit. Free radicals frantically seek electrons in order to pair their unpaired electrons (Nishikimi M 1970). Free radicals cause a chain of reactions loading to consecutive oxidation. These radicals attack on molecules like fat, proteins, DNA, sugar etc. The newly damaged molecule unfortunately becomes a free radical and thus a chain reaction started (Lynch RE and Fridovich 1978). Free radicals are generated during normal metabolism and exposure to environmental insults such as infections agents, pollution, UV light, radiation and so on. These are highly reactive species capable of wide spread, indiscriminate oxidation and peroxidation of proteins, lipids and DNA which can lead to significant cellular damage and even tissue and/or organ failure.

Production of free radicals in the human body

Free radicals and other ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals (Bagchi K, Puri S 1998). Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions. Enzymatic reactions, which serve

as source of free radicals, include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and in the cytochrome P-450 system (Liu *et al.*, 1999) Free radicals can also be formed in nonenzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions.

Endogenous sources of ROS production

- Mitochondria
- Xanthine oxidase
- Peroxisomes
- Inflammation
- Phagocytosis
- Cellular metabolism (electron transport chain)
- Arachidonate pathways
- Exercise
- Ischemia/reperfusion injury
- Stress.

Different free radicals

Superoxide oxygen ($O2^{\bullet -}$ one electron)

It is generated by direct auto-oxidation of O2 during mitochondrial electron transport reaction. Alternatively, O2•is produced enzymatically by xanthine oxidase and cytochrome P450 in the mitochondria or cytosol (Harsh Mohan 2010). $O_2^{\bullet}$ so formed is catabolized to produce $H_2O_2$by superoxide dismutase (SOD) a metalloprotein. It is considered to be the least reactive type of ROS and the most commonly produced free radical in humans. Once it is produced it triggers arapid cascade of events that creates other free radicals.

Hydrogen peroxide (H_2O_2, two electrons)

H_2O_2 is reduced to water enzymatically by catalase and glutathione peroxidase (both in the cytosol and mitochondria). The enzyme glutathione peroxidase also breaks down any peroxides that form on lipids within the body.

Hydroxyl radical (OH^{-}, three electrons)

It is the most reactive of the free radical molecules. It damages cell membranes and lipoproteins by lipid peroxidation. Damage to lipoproteins in low density lipoprotein plays an important role in atherosclerosis. OH^{-} is formed by radiolysis

of water and by reaction of H2O2 with ferrous (Fe^{++}) ions; the latter process is termed as Fenton reaction (Harsh Mohan 2010).

Nitric oxide (NO), a chemical mediator generated by various body cells (endothelial cells, neurons, macrophages etc), combines with superoxide and forms peroxynitrate (ONOO) which is a potent free radical.

Halide reagent (Chlorine / Chloride) released in the leukocytes reacts with superoxide and forms hypochlorous acid (HOCl) a cytotoxic free radical.

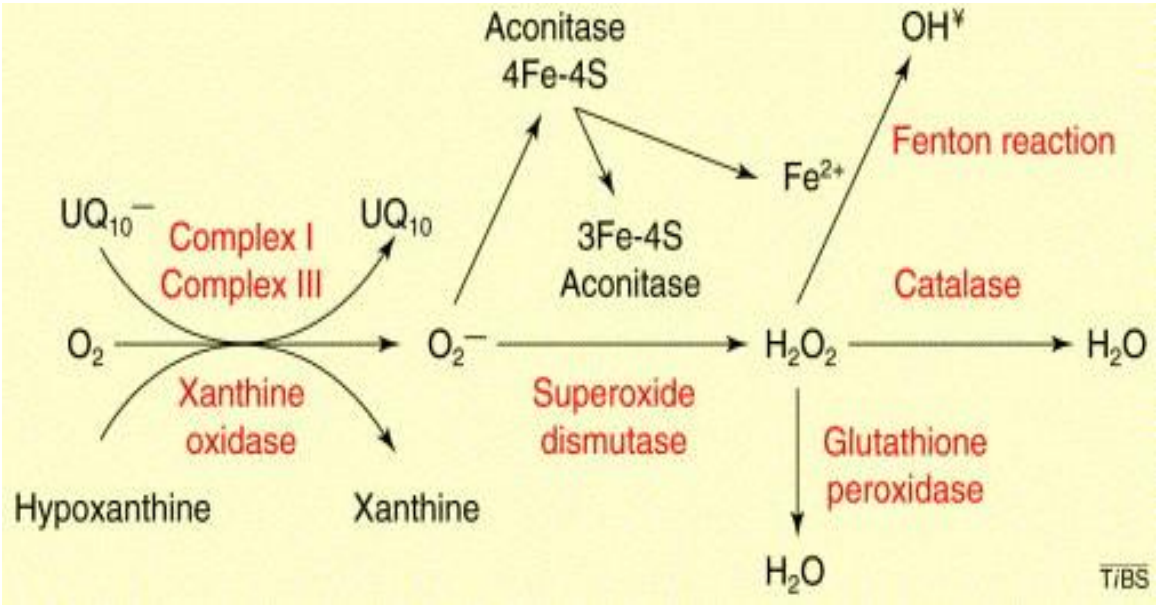


Figure 3: Representation of generation of oxygen free-radical

In human diseases, an increase in free radical activity occurs as a consequence of either primary (excess radiation exposure) or secondary (tissue damage by trauma) mechanism. ROS react with most cellular macromolecules, including proteins, lipids, and DNA. ROS–induced oxidation of proteins can lead to changes in the protein's three-dimensional structure as well as to fragmentation, aggregation, or cross-linking of the proteins. Finally, protein oxidation often will make the marked protein more susceptible to degradation. ROS are a major source of DNA damage, causing strand breaks, removal of nucleotides, and a variety of modifications of the organic bases of the nucleotides which can lead to permanent changes or damage to the DNA, with potentially detrimental effects for the cell (Wu D, 2003).

Sources of free radicals

The sources of free radicals can be endogenous and exogenous in nature. Endogenous sources of free radicals are intracellularly generated from auto-oxidation or inactivation of small molecules. Exogenous sources of free radicals are tobacco smoke, certain pollutants, organic solvents, anesthetics and pesticides. The sites of free radical generation encompass all cellular constituents including mitochondria, lysosomes, peroxisomes, endoplasmic reticulum, plasma membrane and sites within the cytosol (Machlin L J and Bendich 1998). Apart from this, certain medications metabolized to free radical intermediate products also cause oxidative damage within the target tissues. Exposure to radiation results in the formation of free radicals within the target tissues.

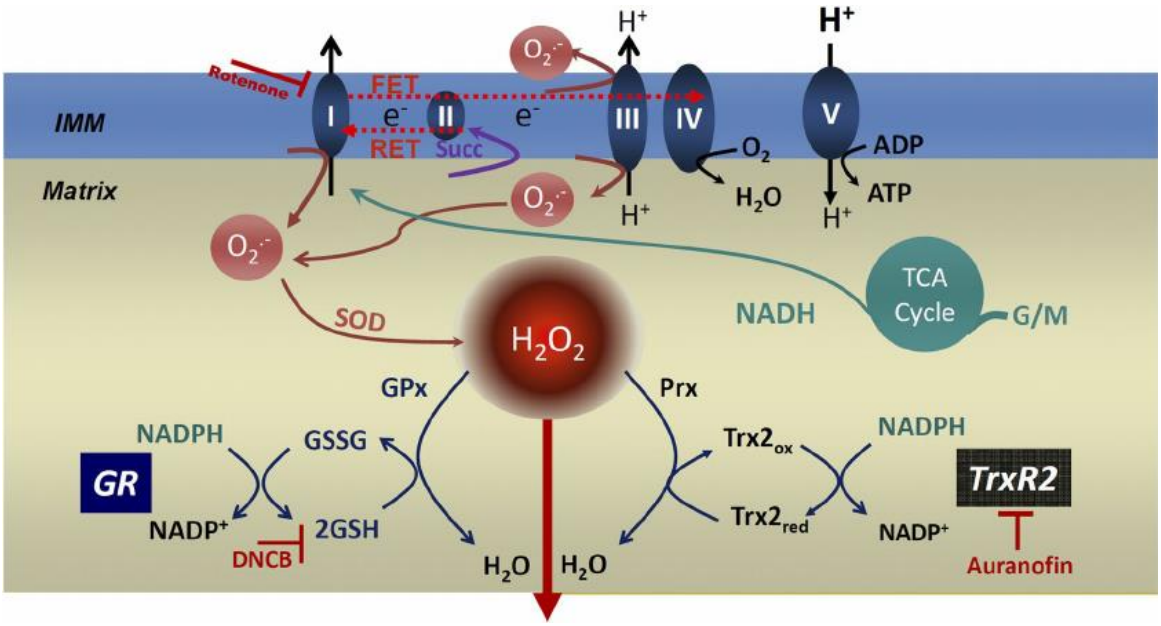


Figure 4: Production and disposal of mitochondrial ROS.

ROS Production in Endoplasmic Reticulum

Similar to mitochondria, ER is another membrane- bound intracellular organelle, but unlike mitochondria, it is primarily involved in lipid and protein biosynthesis. ER when under stress produces ROS mainly by two mechanisms during disulfide bond formation (Bhandary 2003). First, ROS are produced as a by-product during transfer of electrons from protein thiol to molecular oxygen by endoplasmic reticulum oxidoreductin-1 (ERO-1) and protein disulfide-isomerase (PDI) (Bhandary 2003). Alternatively, ROS can be created during misfolding of protein

due to depletion of GSH (Santos 2009), since after GSH is consumed, thiols are repaired enabling them to interact with ERO-1/ PDI and to be re-oxidized (Bhandary 2003). These steps generate consecutive cycles of disulfide bond formation and breakage, with each cycle producing more ROS as a by-product (Higa and Chevet (2012).

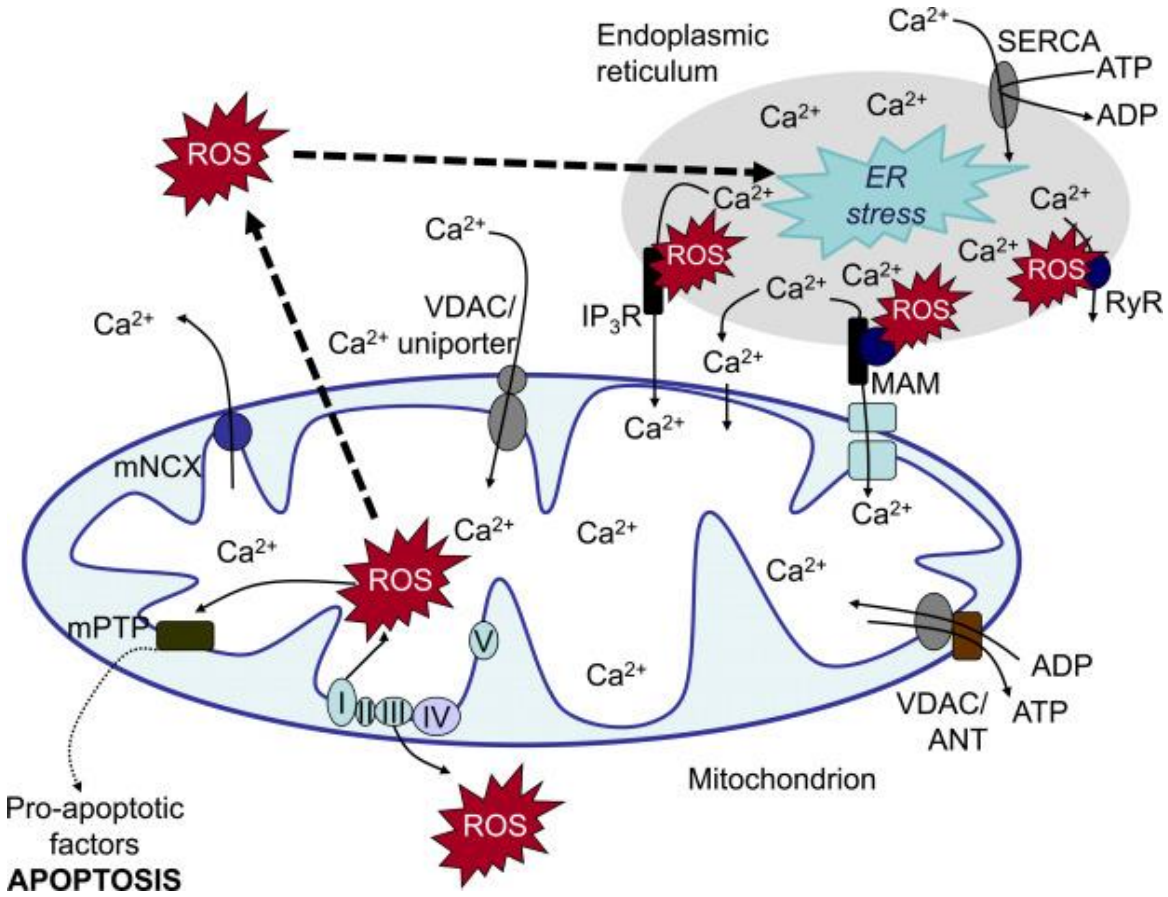


Figure 5: ER and mitochondrial associated reactive oxygen species (ROS) production under ER stress.

Oxidative stress in disease progression

CNS disorders

Calcium is ubiquitous second messenger in cells. The rapid, uncontrolled increase in cystolic calcium levels initiates activation of numerous enzymes that contribute to cell injury.

- Protease activation causes breakdown of cytoskeleton of the neuron.

- lipases damage plasma membrane lipids and release arachidonic acid, which is metabolized by cyclooxygenases and lipoxygenases to yield free radicals and other mediators of cell injury.
- Activation of nitric oxide synthetase (NOS) leads to release of NO and, in turn, the generation of peroxynitrite, a highly reactive free radical, and activated endonucleases damage DNA thereby rendering neurons susceptible to apoptosis.
- Injury to mitochondria leads to energy failure, free radical generation and release of cytochrome C (Cyt-c) from mitochondria; the latter is one of the means by which neuronal apoptosis is initiated.

NO is in fact, a weak free radical that in turn leads to the generation of peroxynitrite which is a ‘killer substance ‘used by macrophages. In cerebral ischaemia NO is probably both friend and foe. It is likely that during a period of focal ischaemia, the vasodilating effect of NO serves to augment collateral CBF. However, in the post ischaemic phase, NO (probably inducible NO of neuronal origin) contributes to neuronal injury (Patel and Drummond 2005).

Cardiovascular diseases

In cardiovascular disease, low density lipoprotein (LDL) oxidation appears to trigger the process of atherogenesis. Oxidized LDL is thought to reduce NO production by the endothelium, which leads to vasoconstriction. Enhanced platelet aggregation is also induced by oxidized LDL (Adams *et al.,* 1999). Low b-carotene concentration in adipose tissues were associated with a high risk of myocardial infarction in current smokers. Vitamin E intakes are associated with lowered risk of angina and mortality from ischaemic heart disease.

Diabetes mellitus

Oxidative stress is crucial to the aetiology of diabetes mellitus and ensuing damage to different tissues and organs. (Kodiha and Stochaj 2012) Hyperglycaemia causes the generation of ROS leading to increased oxidative stress in a variety of tissues. In the absence of an appropriate compensatory response to endogenous

antioxidants, such as Vit-C and E, catalase, glutathione and SOD, oxidative stress dominates, resulting in the activation of stress sensitive intracellular signalling pathways. This plays a key role in the development of late complications of DM as well as mediating insulin resistance (Fardoun 2007).

Pathogenesis of tissue damage

Free radicals which are formed endogenously act intracellularly within the cell and are released into the surrounding area (Machlin and Bendich 1987). Prime targets for free radical reactions are the unsaturated bonds in membrane lipids. Consequent peroxidation results in a loss in membrane fluidity and receptor ailment and potentially in cellular lysis. Free radical damage to sulphur containing enzymes and other proteins culminates in inactivation, cross linking and denaturation. Nucleic acids can be attacked. Subsequent damage to the DNA can cause mutations. Oxidative damage to carbohydrates can alter any of the cellular receptor function including those associated with hormonal and neurotransmitter responses (Machlin and Bendich 1987). Consequences of oxidative stress are adaptation or cell injury, i.e., damage to DNA, proteins and lipids; disruption in cellular homeostasis and accumulation of damaged molecules (Jakus 2000). Prolonged exposure to free radicals, even at low concentration, may result in the damage of biologically important molecules and potentially lead to DNA mutation, tissue injury and disease (Fang, 2002).

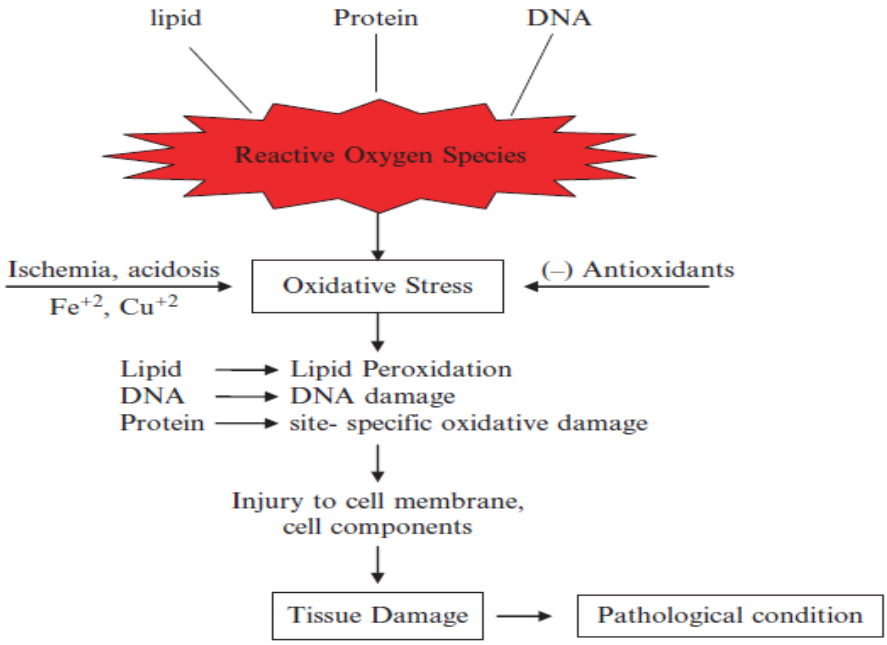
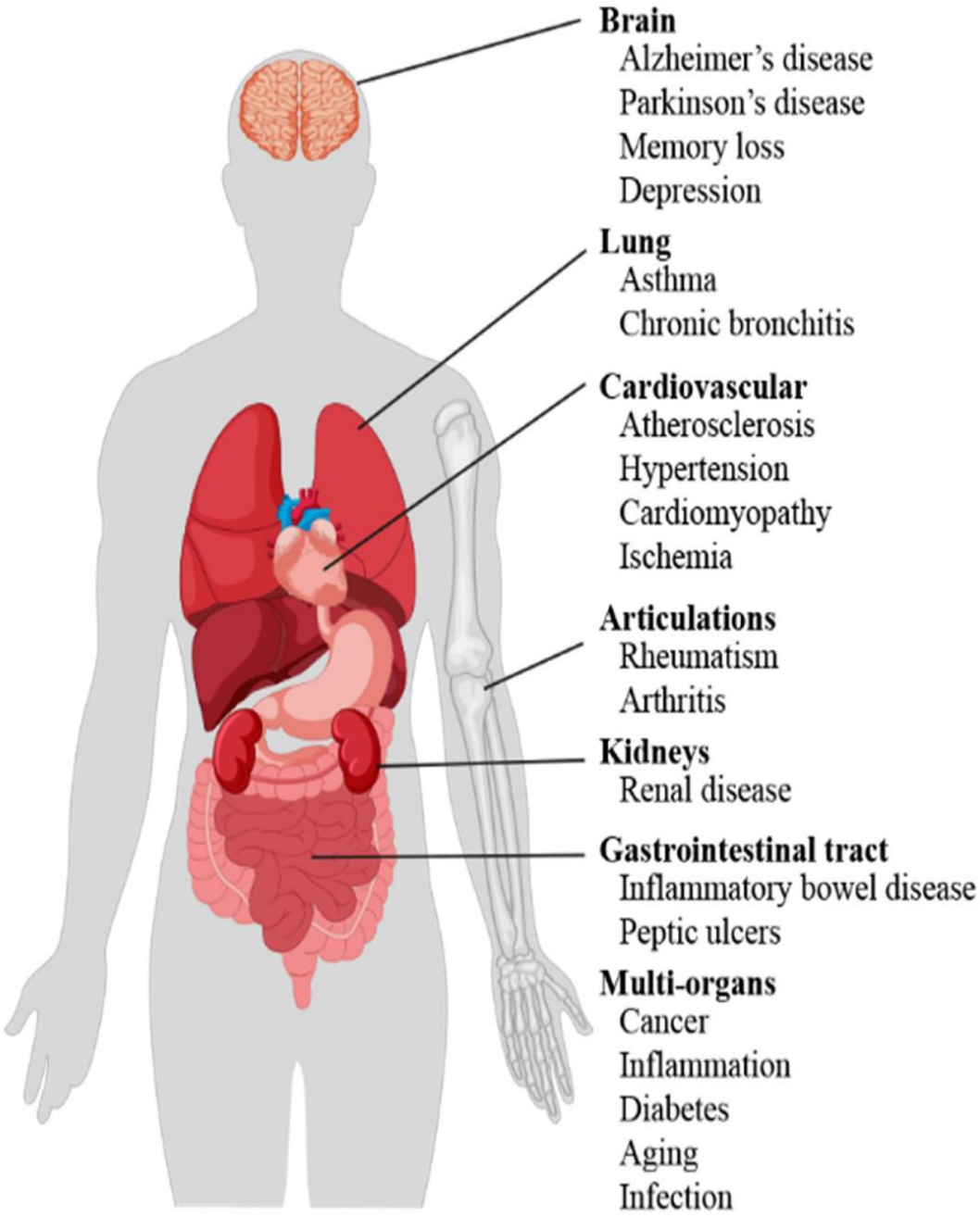


Figure 6: ROS induced tissue damage and disease progression



Figiure 7: Organs affected by oxidative damage

Protection against toxic effects of ROS: Antioxidant systems

Uncontrolled production of ROS often leads to damage of cellular macromolecules (DNA, lipids, and protein) and other small antioxidant molecules (Jakus, 2000). The cells contain important defence systems against free radicals termed as antioxidant enzymes. The main enzymatic scavengers responsible for the prevention of ROS

formation and oxidation are Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and glutathione reductase (GRx). SOD catalyses the dismutation of superoxide to hydrogen peroxide and is the body's primarydefense as it prevents further generation of free radicals. Catalase and glutathione peroxidase detoxify oxygen reactive radicals by catalysing the formation of H2O2. The selenoprotein GPx enzyme removes H2O2 by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme, regenerates GSH from GSSG (oxidized glutathione), with NADPH as a source of reducing power. (Pham-Huy et al., 2008).

Table 1: Different types of defence systems against various Free radicals

Types of free radical (or) oxidants	**Defence system**
Superoxide anion (O_2)	Superoxide dismutase
Hydroxyl radical (OH•)	SOD, Mn-SOD, Cu, Zn-SOD, glutathione
Peroxyl radical (ROO•)	Tocopherols, Ubiquinone
Singlet oxygen (O_2•)	Carotenoid
Hydrogen peroxide (H_2O_2)	Catalase, Se Glutathione Peroxidase
Hydroperoxides (ROO^{-})	Glutathione peroxidase, Glutathione reductase
Transition Metals (Fe+2, Cu+2)	Chelators

Anti-oxidants and their role

The body has several mechanisms to counteract oxidative stress by producing antioxidants, either naturally generated in-situ (endogenous), or externally supplied through foods (exogenous). The role of antioxidants is to neutralize the excess of free radicals, to protect the cells against their toxic effects and to contribute to disease prevention (Pham-Huy *et al.,* 2008) Antioxidants from our diet play an important role in helping endogenous antioxidants for the neutralization of oxidative stress. Each nutrient is unique in terms of its structure and anti-oxidant function (Frei 1997).

Atherosclerosis

Atherosclerosis is a chronic condition characterised by the migration of monocytes into the intima of arteries. These differentiate into macrophages which become lipid loaded by the uptake of low-density lipoproteins. Proliferation and death of these cells contributes to a gradual hardening of the arteries and a narrowing of the vessel lumen that impedes the blood flow. Over time the atherosclerotic lesion can become unstable and suddenly rupture causing formation of a thrombus which can block blood supply to vital organs such as the heart and brain. The result of which is heart attack and stroke, respectively, and these can be collectively termed as cardiovascular disease. Atherosclerosis, hardening of the arteries, is the leading cause of death in the United States, and worldwide. The disease triggers heart attack or stroke, with total annual death of 900,000 in the United States and 13 million worldwide (Hoyert and Xu 2012).

The process of plaque development begins with a lesion in the endothelial layer, allowing LDL, to move from the blood into the intima and becoming oxidized LDL (ox-LDL) by free radicals (FRs). FRs are oxidative agents continuously released by biochemical reactions within the body, including the intima (Cohen A et al 2012). Endothelial cells, sensing the presence of ox-LDL, secrete monocyte chemoattractant protein (MPC-1) (Harrington JR 2000), which triggers recruitment of monocytes into the intima (Osterud B, Bjorklid E 2003). After entering the intima, monocytes differentiate into macrophages, which have an affinity for the ox-LDL (Gui T et al 2012). The ingestion of large amounts of ox-LDL transforms the fatty macrophages into foam cells. Foam cells secrete chemokines which attract more macrophages (Gui T et al 2012). SMCs from the media move into the intima by chemotactic forces due to MCP-1 (Harrington JR 2000), and platelet-derived growth factor (PDGF) (Reape TJ, Groot P 1999).

At the same time that LDL enters the intima, high density lipoprotein (HDL) also enters into the intima, and becomes oxidized by free radicals (McKay C et al., 2005). However, oxidized HDL (ox-HDL) is not ingested by macrophages. HDL helps prevent atherosclerosis by removing cholesterol from foam cells, and by the limiting inflammatory processes that underline atherosclerosis (Barter P 2005). Furthermore, HDL takes up free radicals that are otherwise available to LDL.

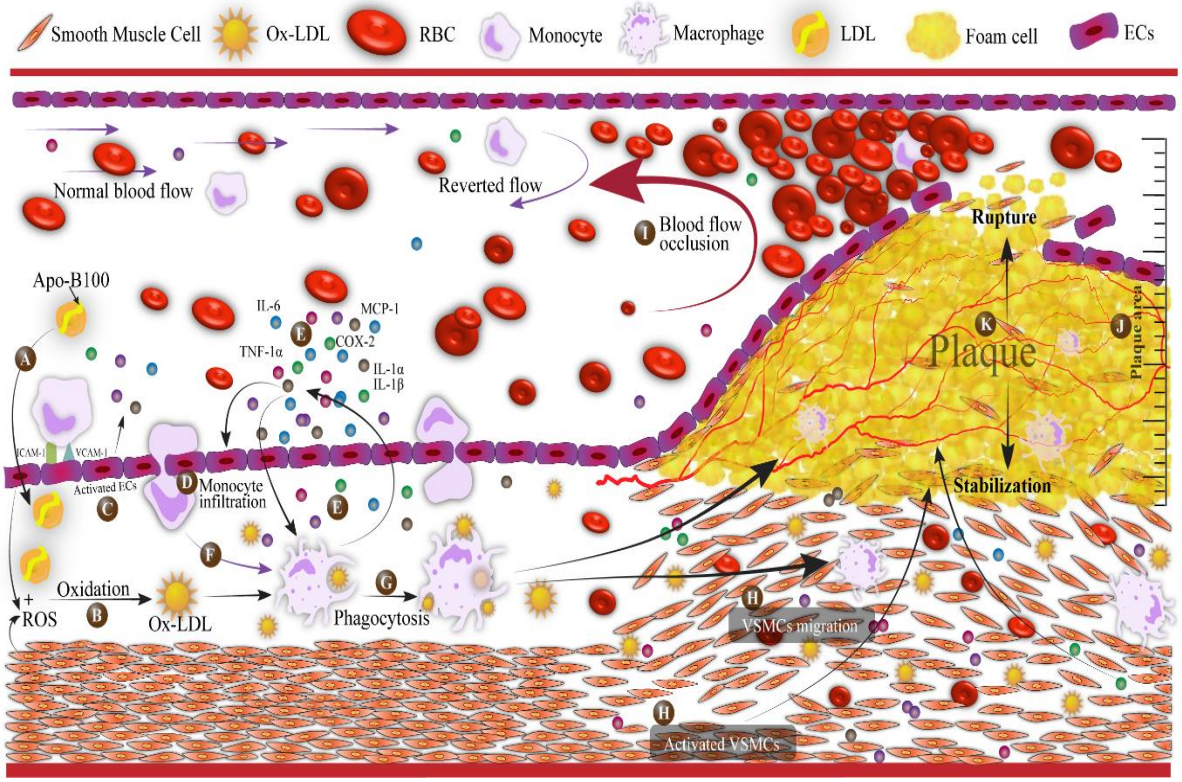


Figure 8: Steps of atherosclerotic progression by Ox-LDL

β-hydroxyl- β-methyl glutaryl CoA reductase (HMG-R)

The rate limiting enzyme of the cholesterol biosynthetic pathway, HMG-R, catalyzes the four-electron reduction of HMG-CoA to CoA and mevalonate, the precursor of isoprenoids. This class of organic molecules consists of modular groups of five carbon atoms and participates in the regulation of diverse cellular functions such as lipid and sterol synthesis, developmental patterning, and protein degradation (Edwards and Ericsson, 1999). Human HMG-R consists of a single polypeptide chain of 888 amino acids. The aminoterminal 339 residues are membrane bound and reside in the endoplasmic reticulum membrane, while the catalytic activity of the protein resides in its cytoplasmic, soluble C-terminal portion (residues 460-888). A linker region (residues 340-459) connects the two portions of the protein.

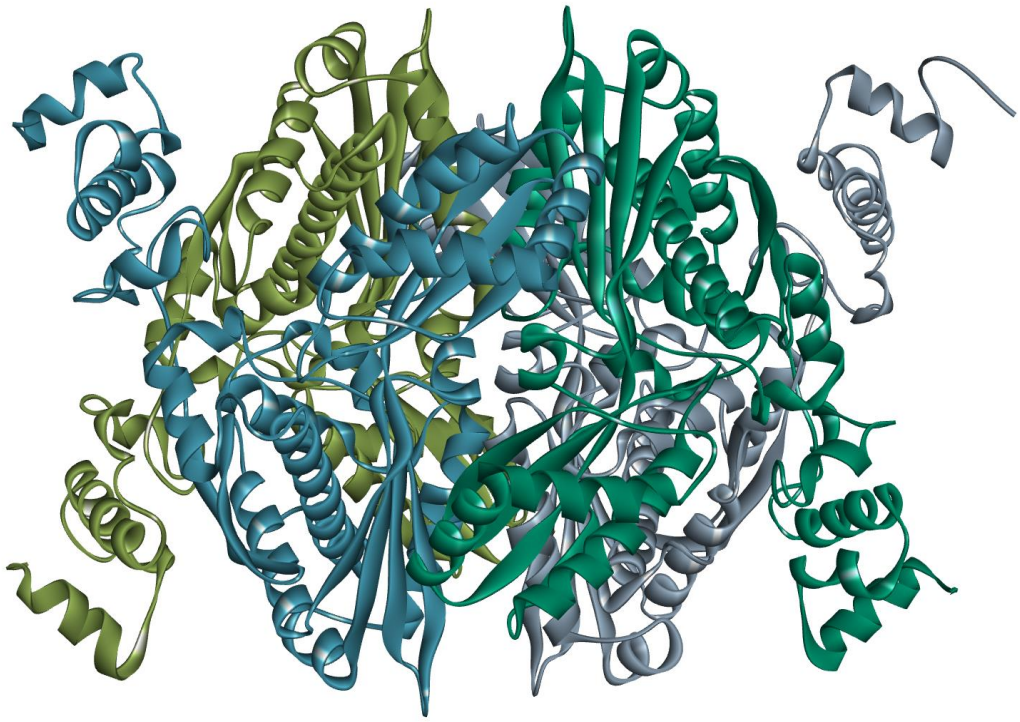


Figure 9: HMG-R contains four identical chains. Chain A-Green; chain B-Yellow, chain C-Gary, chain D-Sky Blue.

The structure of the catalytic portion of HMG-R (residues 460-888) has been described (Istvan et al., 2000) (PDB codes: 1DQ8, 1DQ9, 1DQA). The enzyme forms tetramers with approximate D2 symmetry and overall dimensions of roughly 110 x 80 x 70. The individual monomers wind around each other in an intricate fashion. The monomers are arranged in two dimers (called '1' and '2'), each of which has two active sites. Residues from both monomers (called 'α' and 'β') form the active sites. The interface between dimers is not close to the active sites and the formation of the tetramer does not appear to be involved in substrate binding. The HMG-R monomer reveals a unique structure comprised of three domains: an N-terminal helical domain (N-domain), a large domain (L-domain) whose architecture resembles a prism with a 27-residue α-helix forming the central structural element, and a small domain (S-domain) that is inserted into the L-domain. The S-domain has the fold of an α/β sandwich and a central four-stranded antiparallel β-sheet that resembles the ferredoxins. The HMG-CoA binding site is located in the L-domain, while NADP(H) binds predominately to the S-domain (Istvan and Deisenhofer, 2000).

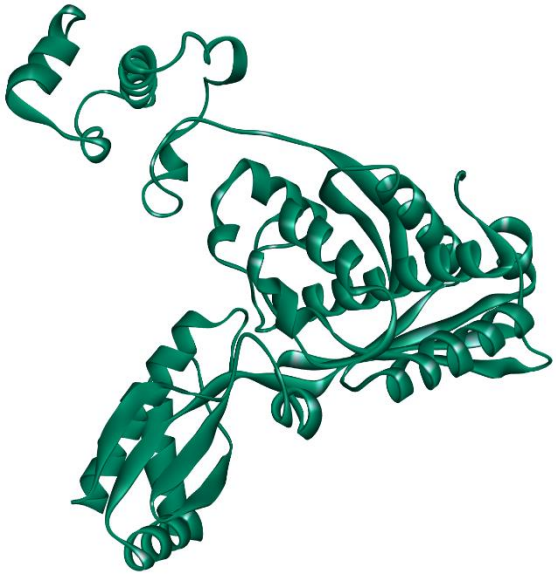


Figure 10: Representation of Chain A of HMG-R.

Plaque Development and Morphology

The normal arterial wall is composed of three defined layers; *the tunica intima*, *the tunica media* and *the arterial adventia* (Saladin 2007). The innermost layer is the tunica intima and this is composed of three further layers; the endothelium, the intima and the basement membrane. The endothelial cells of the endothelium act as a physical and functional blockade from the flowing blood. Endothelial cells also play a regulatory role in many arterial processes including vascular tone and blood pressure regulation, absorption of materials and leukocyte trafficking (Silverthorn 2004). These actions are achieved in part through the numerous mechanoreceptors on the cell surface which respond to endothelial shear stress stimuli (Chatzizisis *et al.,* 2007). This induces a signalling cascade which leads to the activation of transcription factors that ultimately modulates cell function and morphology (Chatzizisis *et al.,* 2007). The intima is normally comprised of a thin layer of loose connective tissue between the endothelium and basement membrane (Saladin 2007). The basement membrane is a layer of elastic tissue. Surrounding the tunica intima is the tunica media. The media consists of layers of smooth muscle cells interspersed with an extracellular matrix composed predominantly of elastin and collagen.

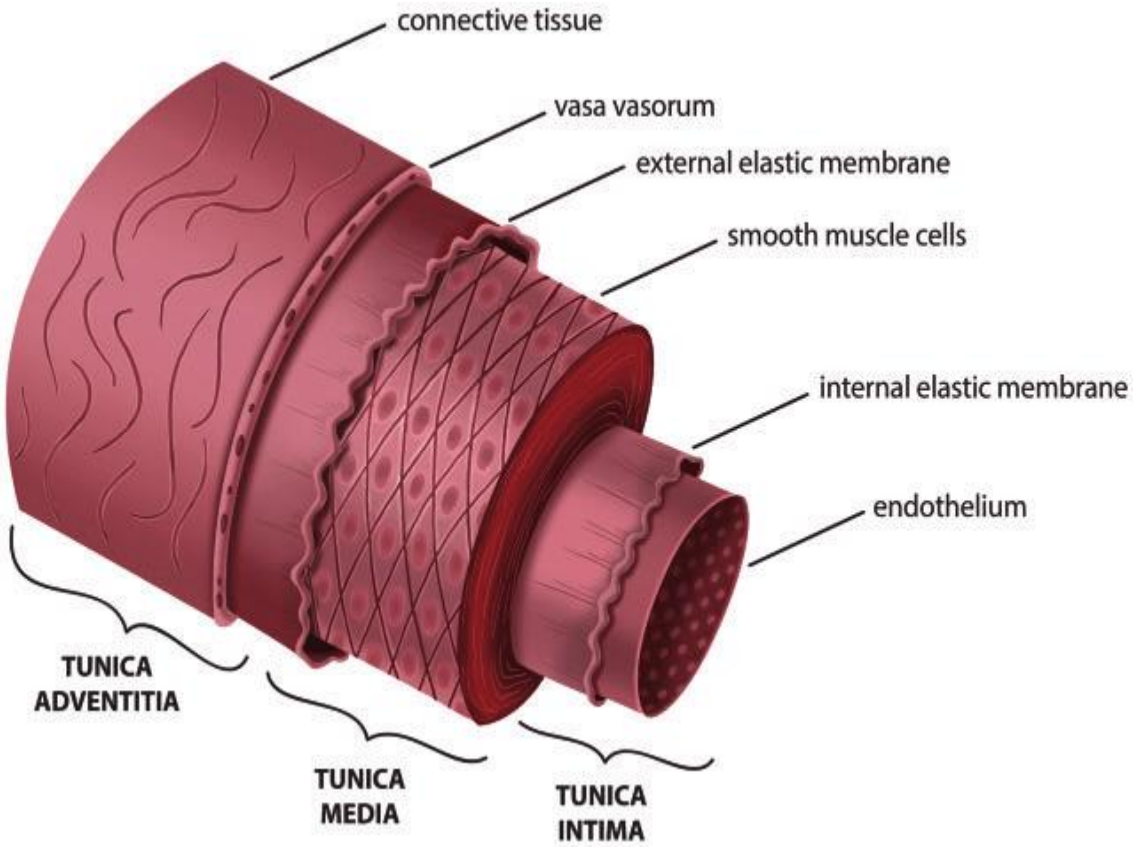


Figure 11: Composition of the normal arterial wall.

Lesion initiation

The first observable stage of atherogenesis is commonly termed as a “fatty streak” due to the sub-endothelial lipid deposition which is visible to the naked eye. Lesions of this type can be commonly observed in children and by the third decade of lifecan occupy as much as one-third of the surface of the aorta on autopsy examination (Stary et al. 1995). However, the lesions are found only in certain specific locations of the aorta. The area of incidence for atherosclerotic lesions correlates strongly with locations in which the blood experiences disturbed flow through the arteries such as at arterial branch points and curvatures (Davies 2000). The transcription factors also promote the accumulation of monocytes via induction of interleukin-8 (Yeh et al. 2004). The disrupted shear stress may also promote arterial smooth muscle cells to produce lipoprotein binding proteoglycans which could contribute to the increased uptake and retention of LDL in the intima of these areas (Libby et al. 2002).

Lesion Progression

Whatever the initiating events, lesion progression is marked by migration and proliferation of smooth muscle cells, the development of an acellular lipid pool which is covered by a fibrous cap and mineral (calcium) deposition. Continued cytokine release by foam cells, macrophages and T-cells along with the release of growth factors by activated platelets leads to the proliferation and migration of smooth muscle cells in the intima. Under normal conditions smooth muscle cells serve to regulate wall tension; however, under these atherogenic conditions, smooth muscle cells develop a synthetic phenotype (Jang et al. 1993). This change is accompanied by further release of self-stimulating growth factors by the smooth muscle cells themselves. This results in a gradual growth of the plaque over time. Throughout the atherogenic process macrophages continue to be recruited into the plaque and differentiate to the foam cell morphology.

Oxidative Stress within the Atherosclerotic Plaque

Oxidative damage is considered to be a key factor in the initiation and progression of atherosclerosis (Libby et al. 2002). Elevated levels of oxidised lipids, proteins and sterols have been detected in all stages of human atherosclerotic plaques showing that oxidative events have occurred or are occurring within the plaque (Upston et al. 2002). Oxidation of LDL is considered to be a crucial component of its unregulated uptake and accumulation by macrophages. It is also toxic to cells thus contributing to formation of the lipid core and decreasing plaque stability (Baird et al. 2005).

Hyperlipidemia

For most primary care providers, hyperlipidemia is defined as elevations of fasting total cholesterol concentration which may or may not be associated with elevated TG concentration. However, lipids are not soluble in plasma, but are instead transported in particles known as lipoproteins. The NCEP created a standard using lipid levels in 2001 that is still the most commonly used clinical classification.

Triglycerides or both are raised in plasma, wherein there is a deposition of lipids mainly in form of esterifies cholesterol in the wall of arteries. Lipids have been

considered as “fats” in the bloodstream, which is commonly divided into cholesterol and triglycerides. However, the cholesterol circulates in the bloodstream which is involved in the structure and functions of cells, whereas, the triglycerides are either used immediately or stored in the fat cells. It causes narrowing and blockage of the arteries and produces mainly heart disease while other diseases include CVD (Cerebrovascular Disease), Renal disease, Liver disease, Peripheral Vascular disease. Hyperlipidemia is not a single disease but a range of disorder with a variety of metabolic disorder, life style disorders and even environmental as well as genetic factors.

It can be caused or influenced by a wide range of other disorders also. Its presence can affect many different organs and systems at the time (Santhoshavangapur et al 2016).

Table 2: NCEP cholesterol thresholds

Triglycerides		**HDL Cholesterol***		**LDL Cholesterol**		**Total Cholesterol**	
<150	Normal	<40	Low	<100	Optimal	<200	Desirable
150 – 199	Borderline high	≥ 60	High	100 – 129	Near or above optimal	200 – 239	Borderline high
200 – 499	High			130 – 159	Borderline high	≥ 240	High
≥ 500	Very high			160 – 189	High		
				≥ 190	Very high		

- Liver is the major site for converting excess carbohydrates and proteins into fatty acids and triglyceride which are then exported and stored in adipose tissue.
- The liver is extremely active in oxidizing triglycerides to produce energy. The liver breaks down many more fatty acids that the hepatocytes need and exports into blood.
- The liver synthesizes large quantities of lipoproteins, phospholipids, amino-acids and cholesterol.

- Hepatocytes are responsible for synthesis of most of the plasma protein (albumin) and also clotting factors.

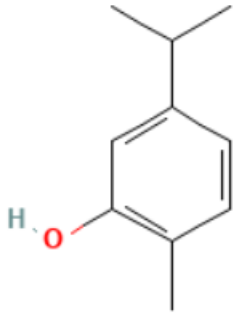
Lipoproteins

Hyperlipidemia is a condition excess of fatty substances called lipids, largely cholesterol and triglycerides, in the blood. It is also called hyperlipoproteinemia because these fatty substances travel in the blood attached to proteins. This is the only way that these fatty substances can remain dissolved while in circulation. American heart association defined hyperlipidemia is a high level of fats in the blood. These fats, called lipids include cholesterol and triglycerides. There are different types of hyperlipidemia depending on which lipid levels are high in the blood (Jain *et al*., 2007).

- Hypercholesterolemia, in which there is a high level of cholesterol
- Hypertriglyceridemia, in which there is a high level of triglycerides, the most common form of fat

The fat-protein complexes in the blood are called lipoproteins. The best-known lipoproteins are LDL (low-density lipoprotein) and HDL (high-density lipoprotein). Excess LDL cholesterol contributes to the blockage of arteries, which eventually leads to heart attack. The higher the level of LDL cholesterol, the greater the risk of heart disease. This is true in men and women, in different racial and ethnic groups, and in all adult age groups. Hence, LDL cholesterol has been labelled the bad cholesterol. In contrast the lower the level of HDL cholesterol, the greater the risk of coronary heart disease. As a result, HDL cholesterol is commonly referred to as the good cholesterol. Low HDL cholesterol levels are typically accompanied by an increase in blood triglyceride levels. Studies have shown that high triglyceride levels are associated with an increased risk of coronary heart disease. Although hyperlipidemia does not cause to feel bad, it can significantly increase the risk of developing coronary heart disease, also called coronary artery disease or coronary disease. People with coronary disease develop thickened or hardened arteries in the heart muscle. This can cause chest pain, a heart attack, or both. Because of these risks, treatment is often recommended for people with hyperlipidemia.

Carvacrol



Carvacrol is a monoterpenoid phenolic compound and also known as cymophenol (2-methyl-5-propan-2-ylphenol) (Wijesundara et al 2018). Carvacrol is present in the essential oil of *Origanum vulgare* (oregano) Carvacrol, which is present in essential oils of Origanum vulgare*, Thymus vulgar*is, *Trachyspermum ammi*, *Lepidium africanum*, and *Citrus bergamia* has been widely studied as an antimicrobial, antiviral, and anticancer agent (Honório et al., 2015; Monte et al., 2014). The essential oil of thyme subspecies contains between 5% and 75% of carvacrol, while Satureja (savory) subspecies have a content between 1% and 45% (Vladić et al., 2016). *Origanum majorana* (marjoram) and Dittany of Crete are rich in carvacrol, 50% and 60–80% respectively (De Vincenzi et al., 2004). It is a colorless oil and it has a characteristic pungent, warm odor of oregano (Ghasemi Pirbalouti et al., 2011)**.** Carvacrol has been classified as GRAS (Generally Recognized As Safe) and approved for food use (EAFUS, 2006; Hyldgaard et al., 2012; European Parliament and Council, 1996). Low toxicity and low cost of production of carvacrol make it an attractive food additive. European Union Food Improvement Agents and Joint FAO/WHO Expert Committee on Food Additives (JECFA), have classified carvacrol as a flavoring agent (NCBI, 2022; Lima et al., 2019)

Traditional application, The ancient Egyptians used thymol and carvacrol as protective agents to preserve the mummies [13]. They were also used as an active additive in food flavoring, perfumes, cosmetics, mouthwash, and some of them have been made for massaging the joints and to treat nail fungi as topical ointments. Drugs formulated from these compounds were administered to care for infections of the mouth and throat and prevent of gingivitis

Biosynthesis

The biosynthesis of carvacrol has been studied. **Figure 1** illustrates a general biosynthetic scheme for enzyme-catalyzed formation of carvacrol and thymol in

green aromatic plants via the mevalonate pathway (Sell, 2010; Nhu-Trang et al., 2006). Briefly, the major steps involve cleavage of glucose to phosphoenolpyruvate followed by decarboxylation and acetylation to acetyl coenzyme A (acetyl CoA) and transformation to the key intermediate, mevalonic acid. The latter is then transformed to γ-terpinene, which then undergoes aromatization to p-cymene and hydroxylation to carvacrol and thymol. Not all intermediates are shown in **Figure 1**. The enzymes and cofactors that catalyze the individual transformations are given in the cited references. The discovery of genes that govern the biosynthesis of carvacrol and other monoterpenes via the γ-terpinene intermediate is currently an active area of research (Mendes et al., 2014).

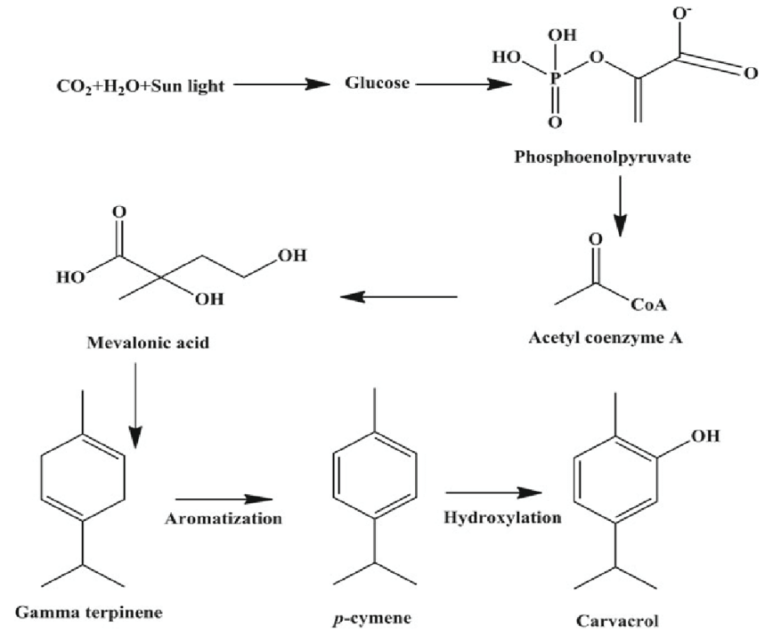


Figure: Biosynthesis of carvacrol through the mevalonate pathway

Yadav and Kamble,(Yadav and Kamble 2009) describe a kinetically controlled synthesis of carvacrol with an 82% yield without the use of solvent (green chemistry) that involves a one-step alkylation of ortho-cresol with propylene or isopropyl alcohol over solid acid catalysts. Synthetic carvacrol may be less expensive than the plant-derived natural form.

Physical Properties.

Carvacrol (liquid; mol wt, 150.22; boiling point, 237−238 °C; density, 0.967 mL/g; melting point, ~0 °C; UV max in 95% ethanol, 277.5 nm; log extinction coefficient

ε, 3.262; pKa phenolic OH group, ~10.9; practically insoluble in water; soluble in ethanol; oral rat LD50, 810 mg/kg; oral rabbit LD50, 100 mg/kg) is a major constituent of oregano oil, widely used as a salad dressing, and numerous other essential oils with concentrations up to ~86% (Yannai 2004; O'Neil, 2013; Baser and Buchbauer 2010). Carvacrol has a pleasant tangy taste and smells like marjoram.10 Carvacrol is considered to be a generally accepted-as-safe (GRAS) compound used commercially as a food flavor (Smith et al., 2005). Standard pure carvacrol for analysis characterized by quantitative NMR (qNMR) is available from a commercial source (Nold, 2014).

Antioxidant activity of carvacrol

Oxidation or oxidative stress caused due to an imbalance between free radicals, generated by several biochemical processes such as light, heat, radiations, transition metals, oxidants and enzymes with detoxifying intermediates, are associated with several chronic disorders in humans, loss of nutrients, as well as the formation of toxic compounds in foods (Escobar et al., 2020; Sharifi-Rad et al., 2018). Free radicals, especially reactive oxygen species (ROS), exert deteriorative effects by abstracting electrons. The main factor that is driving the use of plant-based compounds is their bioactivity, mainly antioxidant activity with diverse modes of action with multiple functions and no side effects (Lawal et al., 2017; Srinivasan, 2016), leading to their being the base of several traditional medicines (Konishi, 2017).

The ability of carvacrol, reduces oxidative stress by scavenging the free radicals and ROS by donating electrons and interrupting the radical chain reaction of lipid peroxidation, stimulating endogenous antioxidative enzymes and augmenting their antioxidant activity (Bagetta et al., 2020; Escobar et al., 2020). However, the rate of lipid oxidation inhibition depends on the nature of the medium. Gursul et al. (2019) have recently evaluated the efficacy carvacrol as antioxidants in microencapsulated walnut oil. The results suggested that fortification carvacrol reduced walnut oxidation. Moreover, encapsulation improved the storability, providing extensive interaction (ability to donate hydrogen) and stabilizing lipid radical. Oregano oils containing carvacrol is being used as antioxidant polyphenols in food packaging. Llana Ruiz-Cabello et al. (2015) evaluated their antioxidant

power individually and combined. The results showed that at higher concentration of thymol, carvacrol and its mixture induced oxidative stress, highlighting the need to optimize the concentration of natural components for applications as antioxidants. Also, reversion of induced oxidative stress by carvcarol, thymol and its mixture (10:1) was demonstrated, due to ability of carvacrol to scavenge free radicals and the activity was dependent on concertation. Lukic et al. (2020) estimated the antioxidant activity of films impregnated with thymol (27.5%), carvacrol (21.2%) and their combination (21.5%). Films created with a combination of thymol and carvacrol improved total phenolic content, even after one month of storage. Also, the film with combined thymol and carvacrol showed the best antioxidant activity due to its synergistic interactions, measured using a DPPH assay and reducing power, with good storage stability.

The antioxidant abilities of carvacrol were similar to commercial antioxidants (Yildiz et al., 2020). Also, the addition of compounds in a dose-dependent manner inhibited thermal oxidation and enhanced hydrolytic stability of refined and stripped corn oils. The application of carvacrol was also evaluated in feeds for rats. They decreased oxidative damage and improved sperm quality (Güvenç et al., 2019). In broiler chickens and fish (Nile tilapia), they were found to have a positive effect on growth, increased antioxidant enzyme activity, reduced lipid oxidation and immunity development (Alagawany et al., 2021; Amer et al., 2018;). Carvacrol and thymol at 150 mg/kg could retard lipid oxidation in broiler meat, improving its quality (Boskovic et al., 2019).

Antimicrobial Properties of carvacrol

The threat of microbial contamination is a critical concern of the food industry (Gonelimali et al., 2018). Additionally, the excess usage of antimicrobials is believed to increase the development of microbial resistance, increasing the incidence of microbial infections (Memar et al., 2017). Hence, the search for natural, often specifically plant-based compounds, showing antimicrobial activity has become a strong contributor towards stopping pathogens from developing antimicrobial resistance (Mahizan et al., 2019). The diverse antimicrobial mechanism across a broad spectrum of microbial groups has been shown for natural compounds (Sychrova et al., 2020). Carvacrol has been widely studied as

an antimicrobial, antiviral, and anticancer agent. Carvacrol is a hydrophobic monoterpene in nature that easily penetrates the cell membranes of bacteria, leading to disruption of cell membrane integrity as well as release of bacterial cell contents (magi et al., 2015). The major mechanism of antimicrobial property of carvacrol are reported to: (i) disrupt bacterial cell membrane; (ii) reduction of biofilm formation; (iii) inhibition of microbial motility; (iv) inhibition of microbial ATP-ases; (v) inhibition of bacterial efflux pump (Kachur & Suntres, 2020).

The antibacterial activities of carvacrol against *S. pyogenes strain* showed growth inhibitory effects against all four (ATCC 19615, ATCC 49399, Clinical isolate, and Spy 1558) tested strains of *S. pyogenes* with the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol against *S. pyogenes* were 125 µg/mL (0.53 mM) and 250 µg/mL (1.05 mM), respectively (Wijesundara et al., 2021). Khan et al., determine the antimicrobial potential and the principal mechanism of action of carvacrol against ESBL *Escherichia coli* isolated from ascitic fluid of a patient having a urinary tract infection. Carvacrol exhibited a minimum inhibitory concentration (MIC) of 450 µg/ml at which it reduced *E. coli* cell counts significantly in a time-dependent manner. Carvacrol completely diminished the growth of E. coli after 2 h of incubation at its MIC (Khan et al., 2017)

Rúa et al. (2019) investigated antimicrobial effects of carvacrol and thymol against 19 different strains of Staphylococcus aureus from different origin. The minimum inhibitory concentration (MIC) was found to be 384.21 µg/ml for carvacrol and 511.84 µg/ml for thymol individually. The Authors have also observed higher MIC for S. aureus strains originating from milk in comparison to meat. The mean minimum bactericidal concentration (MBC) against S. aureus strains was 433.33 µg/ml for carvacrol and 561.64 µg/ml for thymol, while when used in combinedly they exhibited 1013.33 µg/ml MBC. In vitro effectiveness of carvacrol (5 and 10 µg/ml) and thymol (2.5, 5 and 10 µg/ml) against tropical disease Leishmania infantum affecting worldwide populations (Youssefi et al., 2019).

When antibacterial activity of thymol and carvacrol derivatives were tested against five pathogenic microbes, the results suggested that modifying structures resulted in changes of antibacterial activity (Rúa et al., 2019). Action mechanisms of carvacrol and thymol against E. coli were reviewed (Kachur & Suntres, 2020).

Both components showed similar levels of inhibitory effects due to their structural isomerism and the presence of the hydroxyl group.

Effect of thymol and carvacrol on biofilm formation

Biofilm biomass is a mixture of exopolysaccharides, proteins, DNA, and extracellular matrix that has the stabilizing role of biofilm construction (Davey and O'toole 2000). Bacteria in a biofilm are much more resistant to antibiotics than to planktonic status (Mah and O'toole 2001). The plant derivatives can effect on microbial biofilms (Nuryastuti et al., 2009). Several studies described carvacrol inhibited growth of preformed biofilm and interfered with biofilm formation during planktonic growth (Koraichi Saad et al., 2011). Nostro et al. (Nostro et al., 2007) reported carvacrol attenuated biofilm formation of *S. aureus* and *Staphylococcus epidermidis* strains on polystyrene microtitre plates and they suggested it repressed expansion of preformed biofilm and obstructed with the biofilm development during planktonic phase.

Anticancer/antitumor activity of carvacrol

Cancer is regarded as a great threat worldwide, a major cause of morbidity, and globally the second largest cause of mortality (Bouhtit et al., 2021). Caused by uncontrolled multiplication of cells in any part of the body, it can eventually spread (Sarkera et al., 2020). However, there are several causes of cancer, including exposure to carcinogenic compounds and an unhealthy lifestyle (Elbe, Yigitturk, Cavusoglu, Baygar, et al., 2020). The anticancer drugs used for treating cancer patients previously showed high toxicity to the tumour as well as the normal cells (Elbe, Yigitturk, Cavusoglu, Uyanikgil, & Ozturk, 2020). Materials derived from natural sources have shown anticancer properties (cell apoptosis and antiproliferation) and have been approved by several regulatory bodies in natural or semisynthetic forms for cancer treatments (Bouhtit et al., 2021; Lagoa et al., 2020). The phenolic monoterpenes found in plants showed broad biological activities of importance (Brahmkshatriya & Brahmkshatriya, 2013; Shinde et al., 2020). Carvacrol has shown multi-targeting action against cancer cells by inhibiting

viability, oxidation and damaging genetic material (Bagetta et al., 2020), as described in the different studies discussed below.

Carvacrol was evaluated in vitro for its anti-cancer properties (Günes-Bayir et al., 2017). Gastric adenocarcinoma (AGS) cell viability was significantly reduced by carvacrol at a concentration of 10–600 µmol/l. Carvacrol was found to increase the generation of ROS, which was positively correlated with AGS cells. Nonetheless, a pro-oxidant status inducing oxidation stress by ROS induced apoptosis and DNA damage with carvacrol treatment in a dose-dependent manner. Mari et al. (2020) examined cell cycle arrest and apoptosis in breast cancer cells using carvacrol. It exhibited anticancerous impact by inhibiting protein expression, inhibiting proliferation and casuing apoptosis in breast cancer cells. Carvacrol concentration (20 µM) inhibited breast cancer cells, while over that it induced apoptosis (Li et al., 2021).

Elbe, Yigitturk, Cavusoglu, Baygar, et al. (2020) compared the anticarcinogenic effects of thymol and carvacrol. The results suggested that thymol and carvacrol showed cytotoxic activity by reducing cell viability, inducing cell apoptosis and lowering the amount of the ovarian cancer cell line. Thymol was more effective in preventing ovarian cancer cell growth over carvacrol in a dose- and time-dependent manner. However, the exact mechanism was not clear.

Bouhtit et al. (2021) studied the effects of essential oil from *Ptychotis verticillata* and their derivatives, carvacrol and thymol, on cancer cell lines. The essential oil showed cytotoxicity on cancer cell lines at lower concentrations (0.01 and 0.02%) due to higher levels of annexin+/PI-cells having an impact on apoptosis. Synergistic anticancer activity of carvacrol and thymol at 200 and 50 µM, and 300 and 50 µM, respectively, eliminated all cancerous cells. The results indicated that the anticancer effect of carvacrol and thymol and their combination could eliminate the resistant cells. Baranauskaite et al. (2017) reported the anticancerous activity of carvacrol. Inhibition (50%) of cancerous cells was observed using carvacrol at a concentration of 199–322 µM. The results confirmed the antiproliferative activity of oregano extracts.

Antihypertensive activity

Hypertension is the leading cause of several health disorders, mainly strokes causing disability and may be even fatal (Wajngarten & Silva, 2019). Around 1.5 million people are expected to be affected by hypertension and related strokes in Europe by 2025 (Wajngarten & Silva, 2019). Treatment is complicated by the limited efficacy and side effects of single drugs or modern therapeutic medication (Laurent, 2017). Several naturally derived products or compounds have been used for the treatment of hypertension (Al Disi et al., 2016) due to their low cost, easier availability and lack of side effects. Components present in natural compounds are also able to modulate the development of hypertension (Chiu et al., 2020). Phytochemical compounds such as flavonoids, alkaloids, terpenes, phenol acids and polyphenols, found in different natural plants, have shown antihypertensive activity (Cardoso-Teixeira et al., 2020; Micucci et al., 2020).

Carvacrol is natural monoterpenoid phenols. Terpenoid compounds from different natural sources may be used to develop therapeutic agents against hypertension (Cardoso-Teixeira et al., 2020).

Jamhiri et al. (2019) confirmed the antihypertensive activity of carvacrol by inhibiting transient receptors and calcium channels in rat models at a dosage of 50 and 75 mg/kg/day. The compounds also prevented angiotensin induced hypertrophy in vitro. Blood pressure and heart rate were significantly lowered. Lower carvacrol concentration (25 mg/kg/day) could not exhibit the hypertrophic effect. Zhang et al. (2016) reported the role of carvacrol in reducing pulmonary arterial hypertension and oxidative stress using several mechanisms. It also reduced oxidative stress by increasing levels of superoxide and glutathione activity. Thymol and carvacrol, a major active constituent of Thymus linearis, could induce vasodilation by opening the potassium channel and acting on smooth muscles in dose dependent manner (Alamgeer et al., 2018).

Barreto da Silva et al. (2020) reported improvement in antihypertensive effect of carvacrol by incorporating β-cyclodextrin improving drug delivery system. Improvement in antihypertensive activity was observed by inhibiting calcium influx using carvacrol. Additionally, no reduction in blood pressure was observed.

Sargazi Zadeh and Panahi (2017) evaluated the vasorelaxant activity of Trachyspermum ammi extracts rich in thymol (38.1%) and p-cymene (23.1%) with rats. The extracts could relax the aorta in a dose-dependent manner. The

antihypertensive activity was attributed to the blocking of the mechanism regulating the calcium channel. The application of carvacrol at 1, 10 and 20 µg/kg showed a hypotensive impact and decreased systolic and diastolic pressures, heart rate and mean arterial pressure in rats. The impact was found to last up to 2 h (Aydin et al., 2007). The antihypertensive effects of carvacrol encapsulated in cyclodextrin were evaluated. The results showed antihypertensive activity effects, which could be enhanced by encapsulation due to increased bioavailability (Barreto da Silva et al., 2020). The authors indicated that carvacrol could induce vasorelaxation by inhibiting calcium influx.

Immunomodulatory activity of carvacrol

The immune system has an important role by monitoring the invasion of pathogens and providing protection from harmful infections or infectious diseases (Jayaraman & Variyar, 2020). Due to the adverse effects of synthetic drugs, the quest for natural, especially plant-based, compounds as alternatives to modern therapy as modulators of the immune system is increasing (Salehi et al., 2018). Immunomodulators augment the immune response during immunodeficiency or subdue it to weaken the immune system (Tiwari et al., 2018). Plant-derived terpenes have shown the ability to modulate the immune system, as reviewed by Harun et al. (2020).

Phenols from natural compounds interact with dendritic cells, lymphocytes (B or T cells), Th balance, Treg cells, macrophages, and neutrophils interacting with the immune system (Grigore, 2017, pp.75–98). Popa et al. (2020) reported that the immunostimulant activity of terpenes and their derivatives in different plants. Monoterpenes carvacrol was found to increase the white blood cells, measured using bone marrow cellularity, stimulating the humoral immune system (Salehi et al., 2018; Sharifi-Rad et al., 2018). Thymus vulgaris leaf extract, showed strong immune stimulating ability based on a lymphocyte proliferation assay (Al-Dahbi & Awad, 2019). Yin et al. (2017) reported that supplementation carvacrol (25%) could significantly decrease (20%) the most infectious necrotic enteritis caused mortality, alleviate gut lesions and modulate virulence of Clostridium perfringes of poultry. Ability of carvacrol to modulate pathogenicity was highlighted. Similarly, Amer et al. (2018) reported improved immunological capability in Nile

tilapia fingerlings by supplementing feed with carvacrol at 1 ml/kg. Improved immunity was measured by determining lysozyme and catalase activity and immunoglobulins (IgA & IgM) levels.

Additionally, Orhan et al. (2016) reported immunomodulatory activity of carvacrol on ROS production and cytokine inhibition. Marrelli et al. (2018) found carvacrol and its derivatives as major components present in Origanum heracleoticum essential oil. The essential oils showed in vitro anti-inflammatory activity by reducing the production of nitric oxide. Costa et al. (2019) suggested the modulation of inflammation by carvacrol, thymol and essential oils containing monoterpenes, effectively assisting wound healing. Ethanolic extract of Thymus vulgaris, a rich source of thymol and carvacrol, showed positive effects on the immune response (humoral and cellular immune responses) of mice (Al-Dahbi & Awad, 2019).

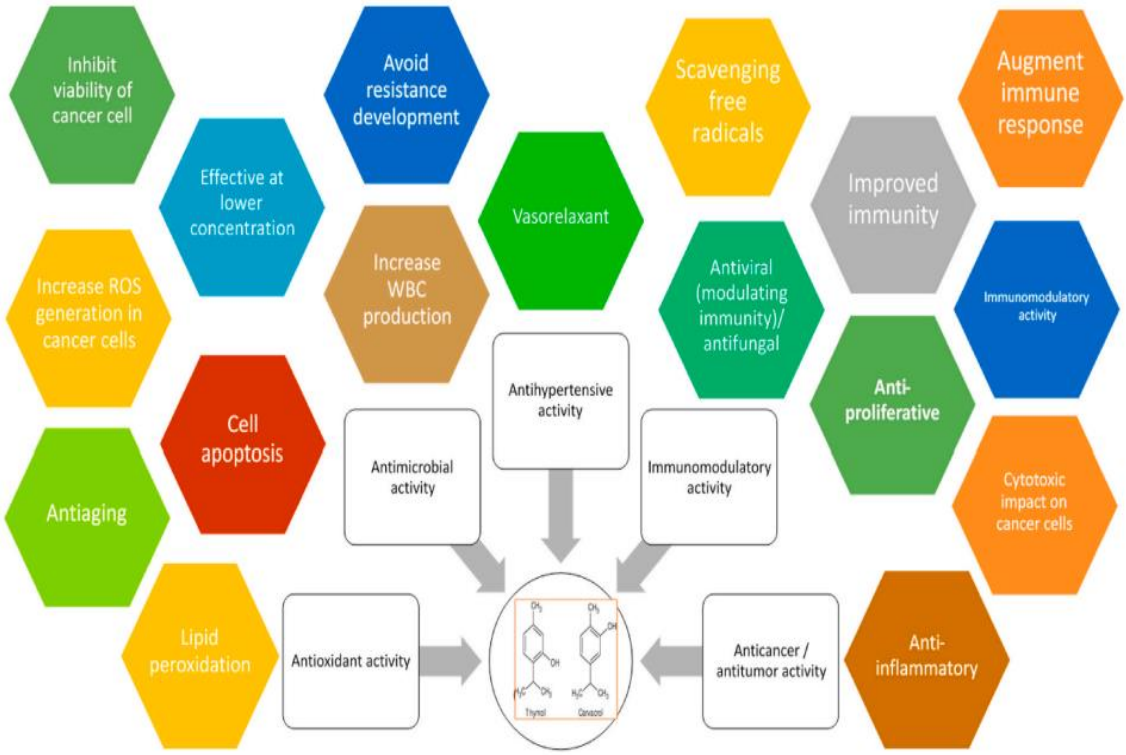


Figure: Biological activity of carvacrol

PubChem

PubChem is a database of chemical molecules and their activities against biological assays. The system is maintained by the National Center for Biotechnology Information (NCBI), a component of the National Library of Medicine, which is part of the United States National Institutes of Health (NIH). PubChem can be accessed for free through a web user interface **(Figure)**. Millions of compound structures and descriptive datasets can be freely downloaded via **File Transfer Protocol** (FTP) PubChem contains substance descriptions and small molecules with fewer than 1000 atoms and 1000 bonds. More than 80 database vendors contribute to the growing PubChem database The File Transfer Protocol is a standard network protocol used for the transfer of computer files between a client and server on a computer network PubChem contains substance descriptions and small molecules with fewer than 1000 atoms and 1000 bonds. More than 80 database vendors contribute to the growing PubChem database (PubChem Source Information).

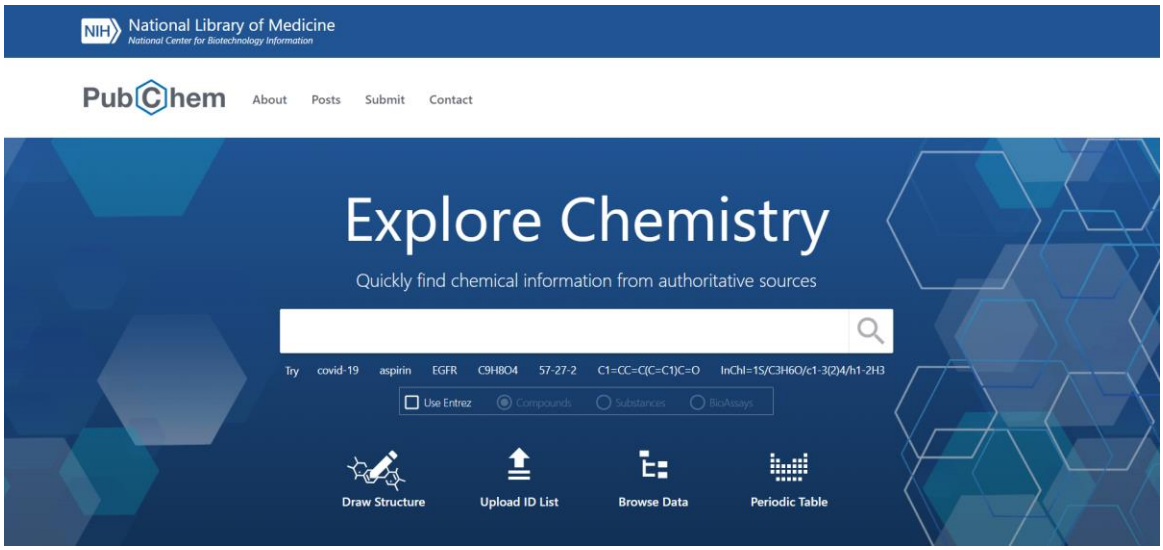


Figure12: Home page of PubChem Data base

PubChem collect information on chemical structures, identifiers, chemical and physical properties, biological activities, patents, health, safety, toxicity data, and many others. Since the launch in 2004, PubChem has become a key chemical information resource for scientists, students, and the general public. Each month the website and programmatic services provide data to several million users worldwide. PubChem mostly contains small molecules, but also larger molecules.

Chemical compounds including drugs Nucleotides including siRNAs and miRNAs, Carbohydrates, Lipids, Peptides, Chemically-modified macromolecules (https://pubchemdocs.ncbi.nlm.nih.gov/about). All the information that PubChem records are contributed by hundreds of data sources. Examples include: Research and development efforts (universities, pharmaceutical companies, etc.), Government agencies, Chemical vendors, Journal publishers, Chemical biology curation efforts (https://pubchemdocs.ncbi.nlm.nih.gov/about)

How Much Data is in PubChem?

PubChem contains the largest collection of publicly available chemical information. Below is a summary of the present record counts in PubChem.

- Compounds: 111 million
- Substances: 278 million
- BioAssays: 295 million
- Tested Compounds: 2,870,244
- Tested Substances: 4,773,804
- BioActivities: 294 million
- Protein Targets: 10,854
- Gene Targets: 22,106

https://pubchemdocs.ncbi.nlm.nih.gov/about

Searching

Searching the databases is possible for a broad range of properties including chemical structure, name fragments, chemical formula, molecular weight, XLogP, and hydrogen bond donor and acceptor count. PubChem contains its own online molecule editor with SMILES/SMARTS and InChI support that allows the import and export of all common chemical file formats to search for structures and fragments. Each hit provides information about synonyms, chemical properties, chemical structure including SMILES and InChI strings, bioactivity, and links to structurally related compounds and other NCBI databases like PubMed. In the text search form the database fields can be searched by adding the field name in square brackets to the search term. A numeric range is represented by two numbers separated by a colon. The search terms and field names are case-

insensitive. Parentheses and the logical operators AND, OR, and NOT can be used. AND is assumed if no operator is used.

RCSB PDB

The Protein Data Bank (PDB) was established as the 1st open access digital data resource in all of biology and medicine It is today a leading global resource for experimental data central to scientific discovery. Through an internet information portal and downloadable data archive, the PDB provides access to 3D structure data for large biological molecules (proteins, DNA, and RNA). These are the molecules of life, found in all organisms on the plane Knowing the 3D structure of a biological macromolecule is essential for understanding its role in human and animal health and disease, its function in plants and food and energy production, and its importance to other topics related to global prosperity and sustainability. RCSB PDB operates the US data centre for the global PDB archive, and makes PDB data available at no charge to all data consumers without limitations on usage (https://www.rcsb.org/pages/about-us/index) **(Figure)**. The Vision of the RCSB PDB is to enable open access to the accumulating knowledge of 3D structure, function, and evolution of biological macromolecules, expanding the frontiers of fundamental biology, biomedicine, and biotechnology. Recognized experts in fields, including but not limited to, structural biology, cell and molecular biology, computational biology, information technology, and education serve as advisors to the RCSB PDB (https://www.rcsb.org/pages/about-us/index)

Figure 13: Homepage of Protein Data Bank (PDB)

Collaborations

Worldwide Protein Data Bank (wwPDB)

The Worldwide Protein Data Bank (wwPDB) was formed to maintain a single PDB archive of macromolecular structural data that is freely and publicly available to the global community. It consists of organizations that act as deposition, data processing and distribution centers for PDB data. As the US Data Center, RCSB PDB biocurates structures submitted from the Americas and Oceania (https://www.rcsb.org/pages/about-us/index)

EMDataBank

EMDataBank provides access to 3DEM density maps and metadata, news, events, software tools, data standards, and validation methods.

Nucleic Acid Database

NDB contains information about experimentally-determined nucleic acids and complex assemblies (https://www.rcsb.org/pages/about-us/index)

Funding

The RCSB PDB is funded by a grant (DBI-1338415) from the National Science Foundation, the National Institutes of Health (National Institute of General Medical Sciences and National Cancer Institute), and the US Department of Energy.

In the past, RCSB PDB was also funded by the National Library of Medicine, the National Center for Research Resources, the National Institute of Biomedical Imaging and Bioengineering, and the National Institute of Neurological Disorders and Stroke (https://www.rcsb.org/pages/about-us/index)

AutoDock

AutoDock is a molecular modeling simulation software. It is especially effective for protein-ligand docking. AutoDock 4 is available under the GNU General Public License. AutoDock Vina is available under the Apache license. AutoDock is one of the most cited docking software in the research community. It is a base for the FightAIDS@Home project run by World Community Grid. In February 2007, a search of the ISI Citation Index showed more than 1100 publications have been cited using the primary AutoDock method papers. As of 2009, this number surpassed 1200. AutoDock is currently maintained by The Scripps Research Institute and Olson Laboratory.

AutoDock is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure. Current distributions of AutoDock consist of two generations of software: AutoDock 4 and AutoDock Vina. AutoDock 4 actually consists of two main programs: autodock performs the docking of the ligand to a set of grids describing the target protein; autogrid pre-calculates these grids (http://autodock.scripps.edu/#WHAT)

AutoDock Vina does not require choosing atom types and pre-calculating grid maps for them. Instead, it calculates the grids internally, for the atom types that are needed, and it does this virtually instantly. autodock also developed a graphical user interface called AutoDock Tools, or ADT for short, which amongst other things helps to set up which bonds will treat as rotatable in the ligand and to analyse dockings.

AutoDock has applications in:

- X-ray crystallography;
- structure-based drug design;
- lead optimization;
- virtual screening (HTS);
- combinatorial library design;
- protein-protein docking;
- chemical mechanism study

AutoDock 4 is free and is available under the GNU General Public License. AutoDock Vina is available under the Apache license, allowing commercial and non-commercial use and redistribution. Click on the "Downloads" tab. And Happy Docking!

OBJECTIVES

1. Literature search for the carvacrol and associated activity with other disease.

2. Retrieval of carvacrol chemical structure from PubChem database and retrieval of target protein β-hydroxyl- β-methyl glutaryl CoA reductase from PDB protein database

3. Protein and ligand's structure minimization via Discovery Studio and ChemOffice

4. To perform *in-silico* molecular interaction study of carvacrol against active pocket of β-hydroxyl- β-methyl glutaryl CoA reductase.

5. *In-vitro* inhibition potential of carvacrol against of β-hydroxyl- β-methyl glutaryl CoA reductase and their enzyme kinetic study

METHODS

In-vitro HMG-R activity

The HMG-R inhibitory activity was assessed by HMG-R assay kit provide by Sigma-Aldrich (St. Louis, MO, USA) contained human HMG-R enzyme (recombinant GST fusion protein expressed in *E. coli*) catalytic domain was used as per the protocol described by manufacturer to assess the HMG-R inhibitory activity of selected OSCs (Iqbal et al. 2014). The human HMG-R enzyme stock solution had a protein concentration of 0.52–0.85 mg/mL with positive control pravastatin was used. To evaluated HMG-R inhibition according to described assay protocol, reactions with 4 µL of NADPH (to get a final concentration of 400 µM) and 12 µL of HMG-CoA substrate (to get a final concentration of 400 µM) in a final volume of 0.2mL of 100mM potassium phosphate buffer, pH 7.4 were initiation (time 0) by adding 2 µL of HMG-R and incubated in Eppendorf biospectrometer at 37ºC in the presence or absence (control) of 1 µL of particular OSCs dissolved in HPLC grade pure water. The NADPH consumption rate was screened spectrophotometrically for every 20 seconds for up to 15 minutes**.**

HMG-R enzyme kinetic study

In order to determine the kinetic properties of HMG-CoA reductase after addition of Carvacrol, the activity was assayed by using various concentrations of HMG-CoA (100, 200, and 300 µM) in the absence and presence of different concentrations of Carvacrol. Km and mode of inhibition was determined by double-reciprocal Lineweaver-Burk plot analysis according to Michaelis-Menten kinetics, and Ki was determined by Dixon plot (Lineweaver and Burk, 1934; Dixon, 1953).

Molecular modelling studies

The PDB structure of the PCSK-9 was retrieved from the Protein Data Bank (PDB ID: 1DQ9) (Brookhaven Protein Data Bank, http:// www.rcsb.org). The .pdb file was energy minimized. The ligands for the active site of HMG-R were also exported in the form of a single sdf files, as well as a separate ligand file of atorvastatin, used as reference drug, was also obtained as .sdf file from PubChem database.

Molecular docking was performed by using AutoDock 4.2 version (Alvi *et al.,* 2017; Ahmad *et al.,* 2021). Brief docking protocol has been elaborated below:

Retrieving Required Ligand and Target .pdb files from major databases

- Retrieving Target.pdb files from major protein databases http://www.rcsb.org/pdb/home/home.do
- Retrieving Ligand.pdb files from major ligand databases http://www.drugbank.ca/ or http://pubchem.ncbi.nlm.nih.gov/

Preparation of Target.pdbqt file

- Open File > Read Molecule > Select and Open Target.pdb (*Created in first step).
- Target molecule will appear on screen > Click on Edit > Click on Hydrogens > Click on Add > Click Polar Only Click OK > Again Edit > Click Charges > Add Kollman Charges > Click OK > Open Grid > Click on Macromolecules > Click on Choose > Click Target > Click Select Molecule > Click OK.
- Open My computer > Open C drive > Open Cygwin > Open home.
- Create new folder and rename it as 1 (or any other shortname).
- Save Target in Folder
  (*In short: save Target.pdbqt in C:\Cygwin\home\1 and after saving macromolecule gets coloured)

Preparation of Ligand.pdbqt file

- Open Ligand > Click Input > Click Open > Change format from .pdbqt to .pdb > Select Ligand > Click Open > Click OK > Again Open Ligand > Click Torsion Tree > Click Detect Root > Again Open Ligand > Click Torsion Tree > Click Set Number of Torsions > Set number of active torsions between 1 to 6 > Click Dismiss.
- Again, Open Ligand > Click Aromatic Carbons > Click Aromaticity criterion.
- Click OK (* If ‘Enter angle in Degrees: 7.5’) > Again Open Ligand > Click Output > Click Save as PDBQT.
- Save Ligand file in C:\Cygwin\home\1(* In the same folder and in same way as Target.pdbqt file

Preparation of Grid Parameter File (a.gpf)

- Open Grid > Click Set Map Types > Click Choose Ligand > Click Ligand > Click Select Ligand > Again Open Grid > Click Grid Box**.** (*We have used X, Y, and Z dimension as 60x60x60. Further X, Y, and Z centre (Centre Grid Box) can be changed according to the requirements but we are taking them as Default).
- Click File > Click Close saving current > Again Open Grid > Click Output > Click Save GPF > Name the File name as a.gpf > Save a.gpf file (.gpf format) in C:\Cygwin\home\1 (* In the same file where Target and Ligand .pdbqt files were saved).

Preparation of Docking Parameter File (a.dpf)

- Open Docking > Click Macromolecules > Click Set Rigid Filename > Go to C:\ Cygwin\ home\ 1 > Select Target.pdbqt > Click Open > Again Docking > Click Ligand > Click Choose > Click Ligand > Click Select Ligand > Click Accept > Again Docking > Click Search Parameters > Click Genetic Algorithm > Click Accept (*Using Default but we can change no. of GA runs).
- Again Docking > Click Docking parameter > Click Accept (*Using Default) > Again docking > Click Output > Click LamarkianGA (4.2) > Name the File name as a.dpf
- Save a.dpf file (dpf format) in C:\Cygwin\home\1
  (* In the same file where Target and Ligand pdbqt and a.gpffiles were saved) At last four files named

  Target.pdbqt, a.gpf
  Ligand.pdbqt, a.dpf

  are present in the C:\ Cygwin\ home\1

Using Cygwin for Molecular Docking

Open Cygwin (*By clicking icon on the desktop) Use these commands highlighted in brown font color by copy and paste in Cygwin and press enter after each command:

(cd..) cd<space>..

(ls) ls<space>

(cd 1) cd<space>1(or foldername)<space>

(ls) ls<space>

(autogrid4.exe -p a.gpf -l a.glg &)

autogrid(tab)<space>-p<space>a.gpf<space>-l<space>a.glg &

(tail -f a.glg &) tail<space>-f<space>a.glg<space>&

(autodock4.exe -p a.dpf -l a.dlg &)

autodock(tab)<space>-p<space>a.dpf<space>-l<space>a.dlg &

(tail -f a.dlg &) tail<space>-f<space>a.dlg<space>&

After Successful Completion

Copy Target.pdb file in C:\Cygwin\ home\1

Copy and paste the following commands in Cygwin Window and press enter after each command:

(grep '^DOCKED' a.dlg | cut -c9- > a.pdbqt)

(cut -c-66 a.pdbqt > a.pdb)

(cat Target.pdb a.pdb | grep -v '^END ' | grep -v '^END$' > complex.pdb)

- Close Cygwin Window > Click OK

Analyzing results and Retrieving Ligand-Enzyme interaction complex .pdb

Analyzing Results

- Open AutoDock > Click Analyse > Click Docking > Click Open > Select a.dlg > Click Open > Click OK > Again Analyze > Click Conformations > Click Play > Click & > Click show information >Click this sign to observe each conformation from 1 to 10
- Note the confirmation showing best down binding energy and inhibition constant (*In our case 10 conformation was best with binding energy (ΔG) as -5.75 and inhibitionconstant (Ki) as 60.87 μM)

Retrieving Ligand-Enzyme interaction complex .pdb

- Open C drive > Open Cygwin > Open home > Open 1 > Open complex.pdb in Discovery Studio Visualizer.
- Select all other complexes and delete them except the best (*In our case Complex model 10 was best as conformation 10 was showing best results in our case).
- Click Scripts > Click Ligand Interactions > Click Show Ligand Binding Site Atoms >Right Click on Complex > Click Label >Select Object: AminoAcid > Select Attributes: 1 Letter & ID insertion code > Click OK

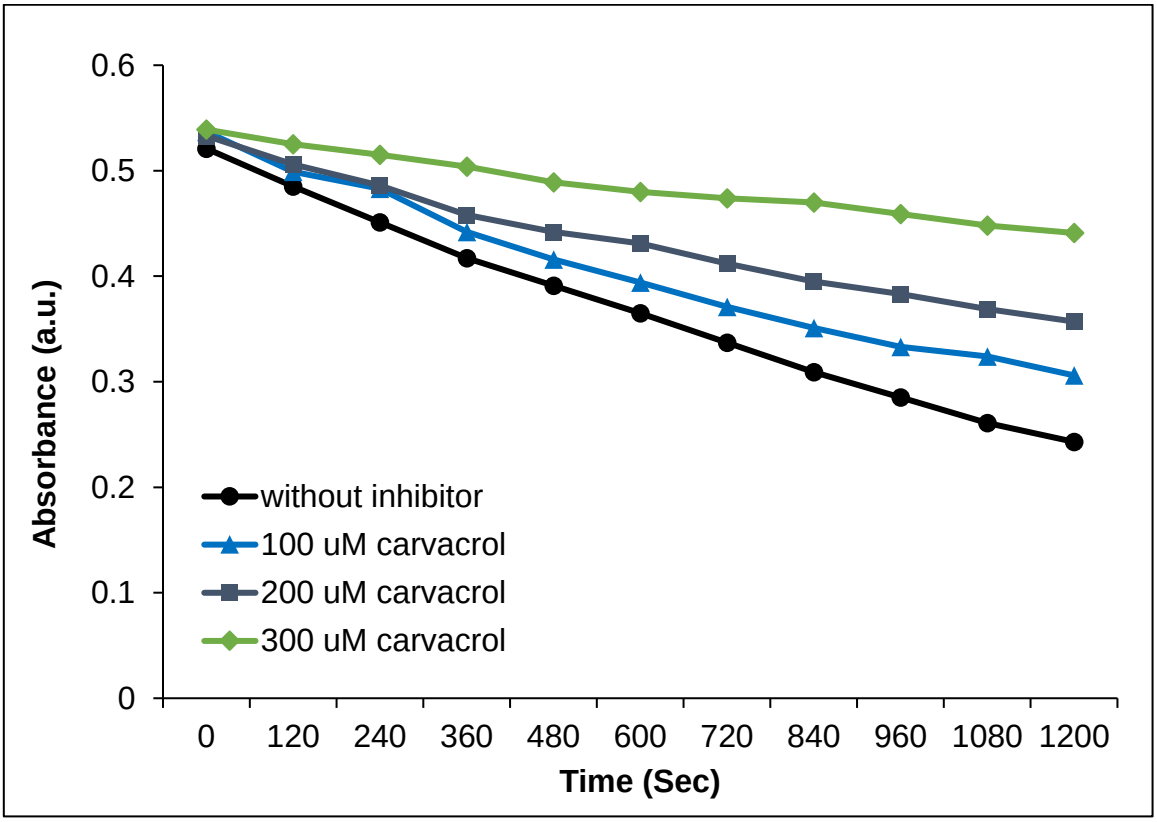


Figure 14: In-vitro HMG-R inhibitory activity time scan of different concentration of carvacrol.

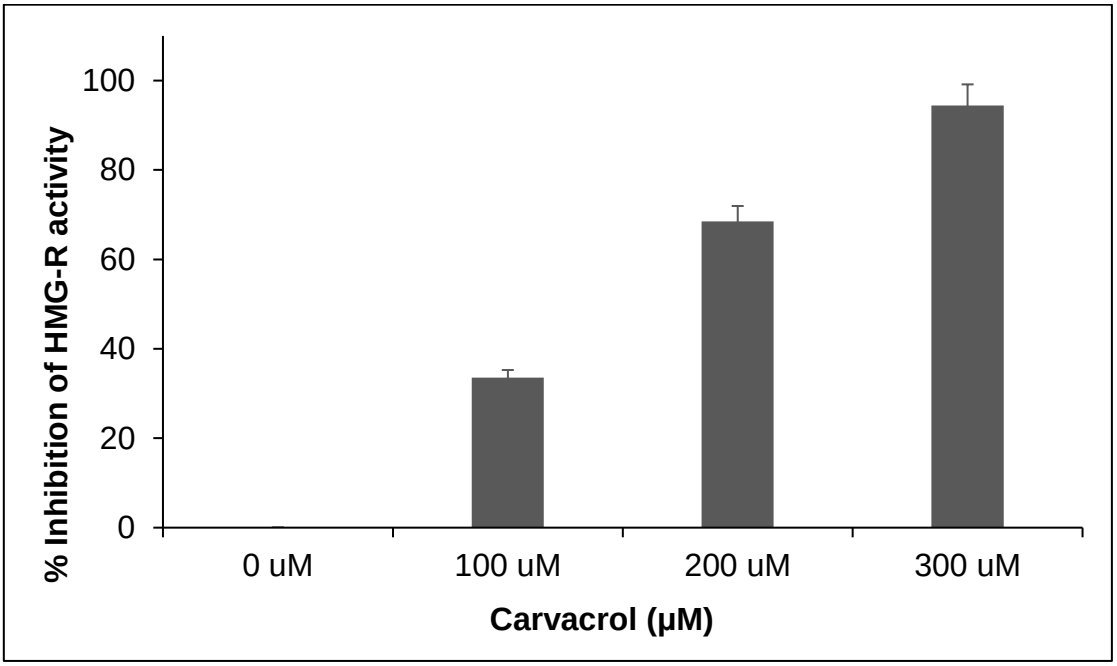


Figure 15: Percent inhibition of HMG-R with different concentration of carvacrol

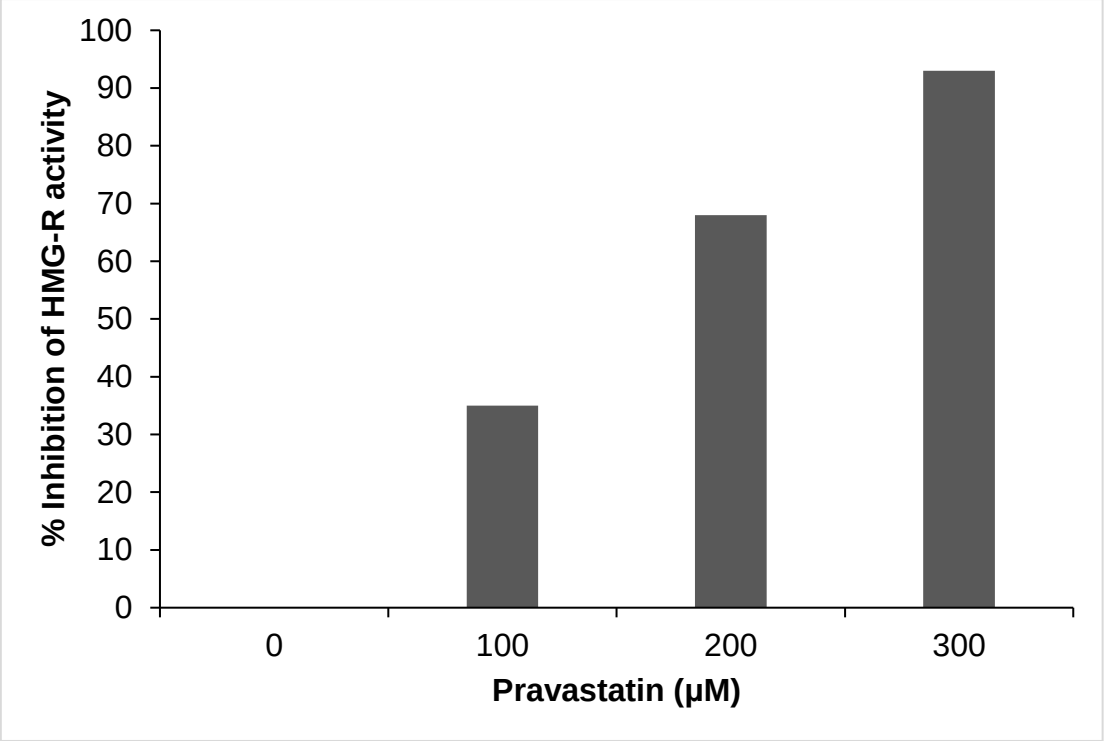


Figure 16: Percent inhibition of HMG-R with different concentration of standard drug pravastatin

Table 4: *In-silico* molecular binding profile of test compound carvacrol and standard drug atorvastatin against HMG-R

S. No.	Compound	ID	Binding Energy (ΔG)	Inhibition Constant
1.	Carvacrol	10364	-4.54	467.04 µM
2.	Pravastatin*	54687	-5.59	128.75 µM

*Standard drug

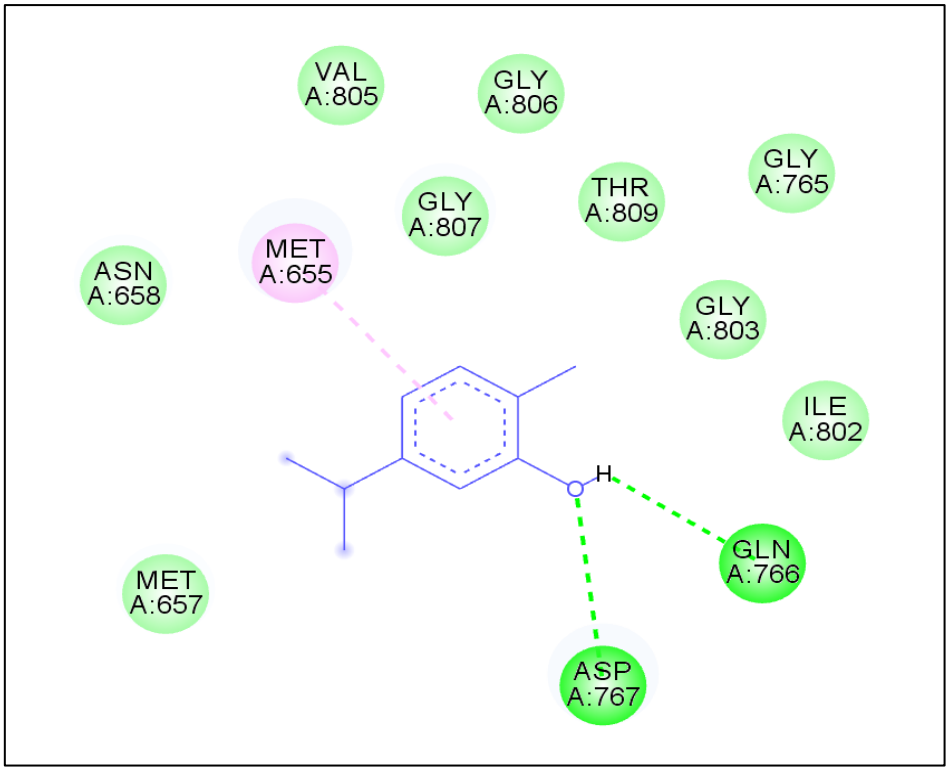


Figure 17: Molecular 2D-interaction of carvacrol (CID: 10364) with the active pocket of β-hydroxyl- β-methyl glutaryl CoA reductase (1DQ9).

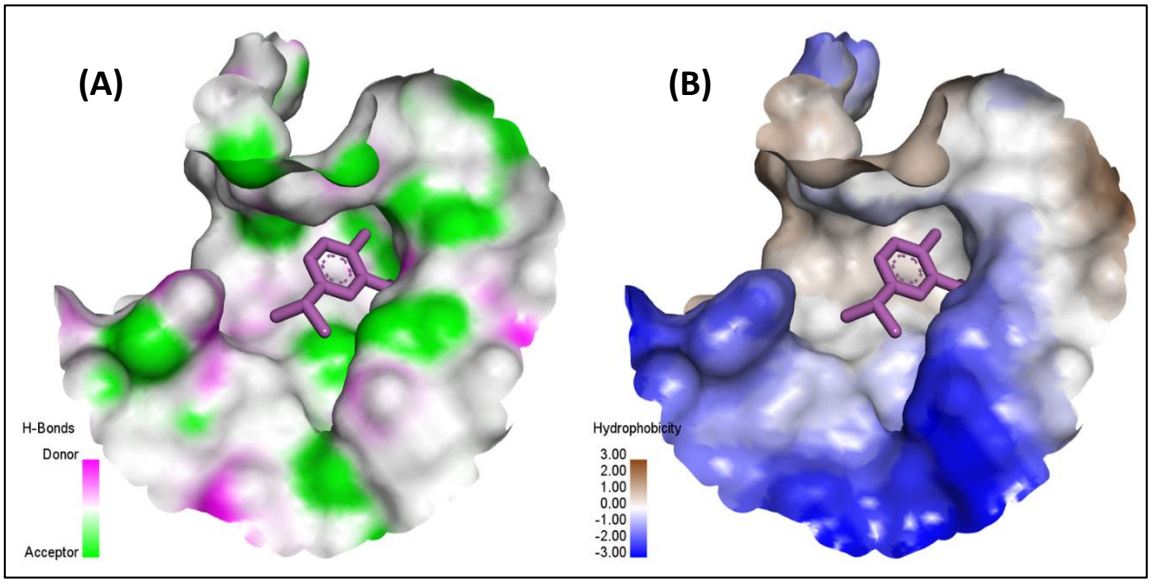


Figure 18: Panel (A)- Formation of hydrogen bonds to stabilize the carvacrol-HMG-R-complex; Panel (B)- Hydrophobic surface around carvacrol-HMG-R-HMG-R complex.

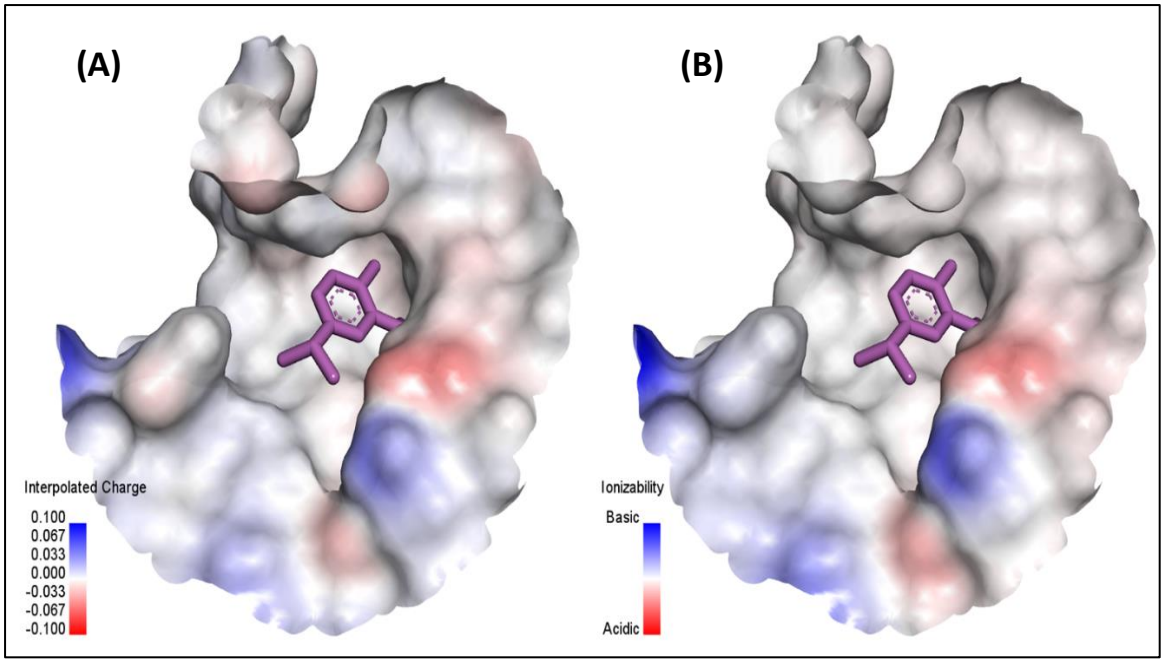


Figure 19: Panel (A)-Formation of interpolated charges to stabilize the carvacrol-HMG-R complex; Panel (B)-ionizability surface around carvacrol-HMG-R complex

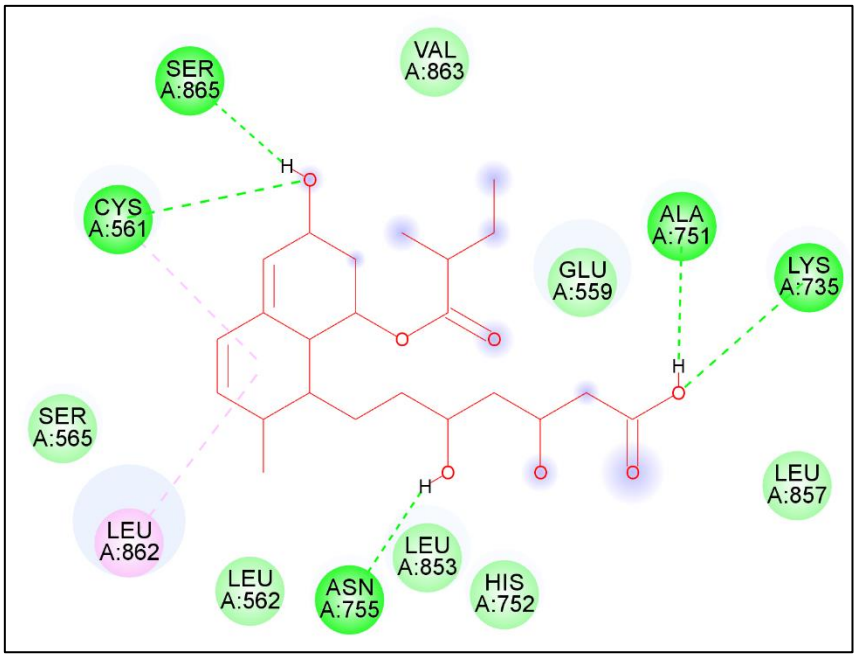


Figure 20: Molecular 2D-interaction of standard drug Pravastatin (CID: 54687) with the active pocket of β-hydroxyl- β-methyl glutaryl CoA reductase (1DQ9).

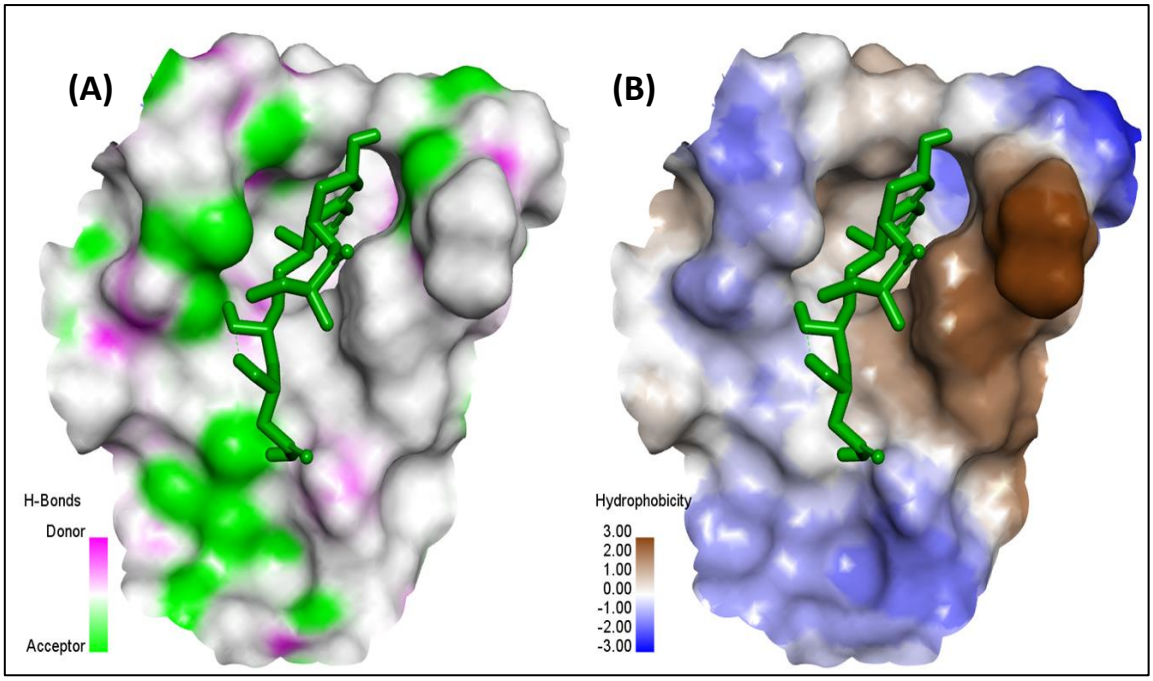


Figure 21: Panel (A)-Formation of hydrogen bonds to stabilize the Pravastatin-HMG-R complex; Panel (B)-Hydrophobic surface around Pravastatin-HMG-R complex.

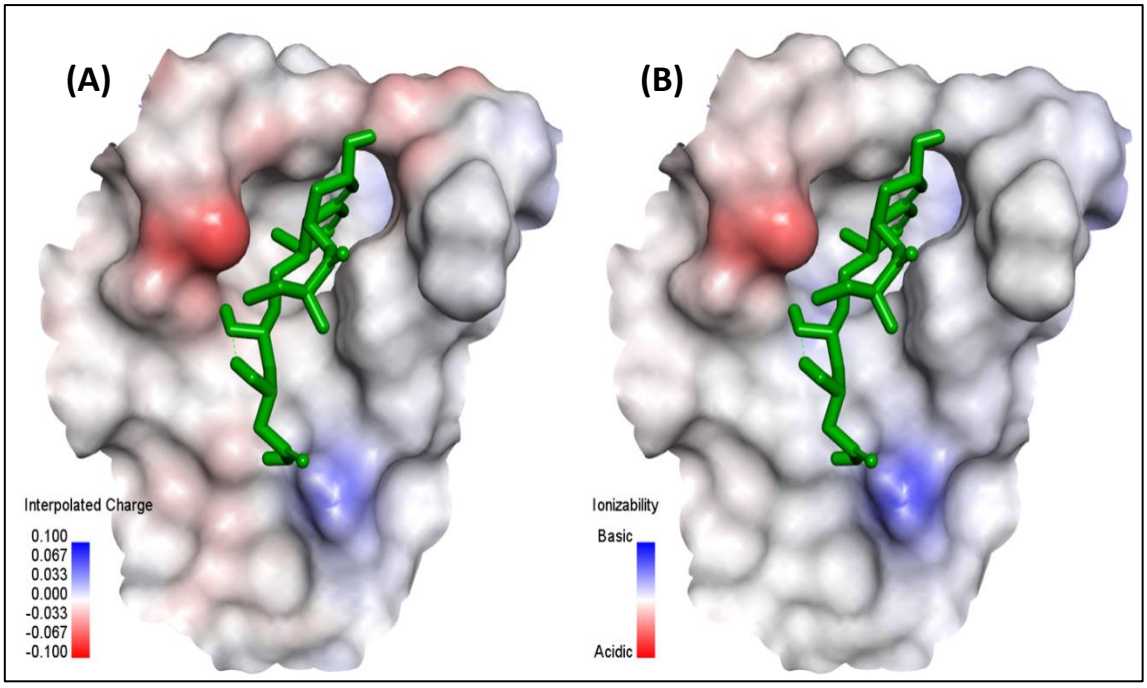


Figure 22: Panel (A)-Formation of interpolated charges to stabilize the Pravastatin-HMG-R complex; Panel (B)- ionizability surface around Pravastatin-HMG-R complex

Table 5: Molecular interaction studies of carvacrol and Pravastatin against the active pocket of HMG-R.

Compound	**Binding Energy (ΔG) Kcal/mol**	**Residues involved**
Carvacrol	-4.54	Met655, Met657, Asn658, Gly765, Gln766, Asp767, Ile802, Gly803, Val805, Gly806, Gly807, Thr809.
Pravastatin*	-5.59	Leu536, Glu559, Cys561, Ser565, Leu857, Lys735, Ala751, His752, Asn755, Leu853, Leu862, Val863, Ser865,

*Standard drug

DISCUSSION

The prevalence of hyperlipidemia, a disorder of lipid metabolism and one of the major risk factors responsible for cardiovascular diseases (CVD), is currently increasing at a striking rate throughout the world (WHO 2015). Therefore, regulating the lipid metabolism and decreasing the higher levels of serum total cholesterol, triglycerides and LDL cholesterol are considered to be quite advantageous for the therapeutic approaches of CVD (Derosa et al. 2006). ASCVD is a class of complex multifactorial disease, which develops in the arterial wall in response to various stimuli and results in excessive inflammatory and fibro proliferative reactions (Mendis et al., 2011; Alvi et al., 2015). The underlying mechanisms vary depending on the type of ASCVD. Atherosclerotic lesions can develop as early as the second decade of life and progress into severe clinical disease with atherogenic plaques over time. The formation of plaque in the arterial intima may be due to hyperlipidemia which refers to the imbalance between various lipids and lipoproteins i.e., total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and apoB100 containing particles such as low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein cholesterol (VLDL-C). Among these cholesterols, LDL-C is the major driver of ASCVD onset worldwide (Alvi et al., 2017a; Alvi et al., 2015).

Therapeutic approaches against ASCVD risk reduction include the implication of statins that specifically and competitively inhibit the enzymatic activity of 3-hydroxy-3-methylglutaryl Co-A reductase (HMG-R), which is the rate limiting enzyme of the hepatic cholesterol biosynthesis (Alvi et al., 2016). However, long term implication of these medications has lots of side effects and may incurve problems in terms of toxicity and cost. Therefore, drugs derived from natural products could be a good alternative in the treatment and management of atherosclerosis. Currently, there is renewed interest in the herbal medications and functional foods modulating physiological effects in the prevention and cure of ASCVD as they possess slight or no side effects with respect to commercially available synthetic drugs, statins. In this order, an array of recent studies has demonstrated the role of various medicinal plants and their therapeutic natural products against HMG-R activity to combat hypercholesterolemia (Iqbal et al., 2014a; Iqbal et al., 2014b; Alvi et al., 2016; Iqbal et al., 2015; Alvi et al., 2017a; Alvi

et al., 2017b).Dietary approach is the initial step in the management of dyslipidemia, and many people with dyslipidemia are using garlic as an alternative medicine to normalize their raised lipid levels.

Therefore, investigation for other possible natural therapeutic agents, which are non-toxic, free radical quencher and are capable to inhibit HMG-CoA reductase activity, could be an important therapeutic approach in treatment and management of hypercholesterolaemia. In this order, multiple plants derived natural compound named Carvacrol, has long been used in perfumes and cosmetics and is considered as one of the important medicinal agents and provides a useful source of new therapeutics (Müller et al., 2008; Mączka et al., 2020; Lir et al., 2020). It has numerous biological activities i.e., anti-inflammatory, antimutagenic, antidiabetic, anticancer, and anti-bacterial activity Mączka et al., 2020; Lir et al., 2020).

The 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA) is converted into mevalonate in the presence of HMG-R in hepatocytes, which is a rate limiting element in cholesterol biosynthesis. The best-known HMG-R inhibitor is statin, which is competitively inhibit HMG-R enzyme. Statins interact with the enzyme's active site and cause a conformational change within the structure, reducing its activity. Furthermore, statins have a binding affinity for HMG-R that is then thousand times greater than the substrate (HMG-CoA), blocking the enzyme from acting and reducing the intracellular synthesis of cholesterol. Statins have a tremendous effect on cholesterol reduction because the majority of the circulating plasma cholesterol forms from internal synthesis in hepatocytes instead of diet (Ahmad et al. 2020; Ahmad, Alvi, and Khan 2019;).

The *in-vitro* HMG-R inhibitory activity was performed. The results indicated that the carvacrol inhibited the in-vitro HMG-R activity in a concentration dependent manner with an IC_{50} value 146.92 ± 3.5 µM. On the other hand, pravastatin was used as standard HMG-R inhibitor which substantially restricted the activity of HMG-R with an IC_{50} Value 153.22 ± 2.1 µM. This study was further validated by the *in-silico* molecular docking approach. We performed computer added molecular modelling strategy to evaluate the anti-hyperlipidaemic potential of carvacrol by targeting HMG-R active pocket. We observed that the carvacrol intensely engaged to the binding region of the HMG-R crystal structure, having binding energy (ΔG) -4.54 kcal/mol (Table 5). with the 12 amino acid residues

(Met655, Met657, Asn658, Gly765, Gln766, Asp767, Ile802, Gly803, Val805, Gly806, Gly807, Thr809.) with 2 hydrogen bond Asn766, and Asp767 and 9 Van der Waals interaction.

On the other hand, the active pocket of HMG-R was also occupied by standard Pravastatin (ΔG: -5.59 Kcal/mol) along with interacting amino acid residues (i.e., Leu536, Glu559, Cys561, Ser565, Leu857, Lys735, Ala751, His752, Asn755, Leu853, Leu862, Val863, Ser865,), with 5 hydrogen bonds with Cys561, Lys735, Ala751, Asn755, and Ser865, as well as 6 Van der Waals contacts, when compared to the pravastatin interaction with HMG-R active pocket. Our in-silico results depicted clearly that carvacrol has potent inhibitors of HMG-R as carvacrol compete with pravastatin for binding at the same active pocket. From now, as we know that there are lots of adverse effects linked with synthetic blocker of HMG-R activity. carvacrol may be encouraged for further drug development process and to initiate natural alternative treatment approach in the management of CVD.

CONCLUSION

ASCVD is a class of complex multifactorial disease, which develops in the arterial wall in response to various stimuli and results in excessive inflammatory and fibro proliferative reactions. Therefore, regulating the lipid metabolism and decreasing the higher levels of serum total cholesterol, triglycerides and LDL cholesterol are considered to be quite advantageous for the therapeutic approaches of CVD. In this order, carvacrol, has long been used perfumes, cosmetics and is considered as one of the important therapeutic agents and provides a useful source of new therapeutics. Carvacrol has been found to show greater effects therapeutic modulations against various metabolic disorders. Therefore, we analysed their potential to inhibit the activity of HMG-R via molecular modelling studies. Our results from the present study demonstrated that carvacrol interact with and occupy the active pocket of HMG-R (-4.18 Kcal/mol), while the pravastatin score the binding energy -5.59 Kcal/mol. On the other hand, the *in-vitro* HMG-R inhibitory activity was performed which substantially restricted the activity of HMG-R with IC_{50} value of carvacrol and standard drug pravastatin was observed 146.92 ± 3.5 µM and 153.22 ± 2.1 µM respectively. Based on above findings, we concluded that carvacrol may be promoted to fight with cholesterol induced oxidative stress and hypercholesterolemia in the patients suffering with inadequate lipid lowering with statins, the classical HMG-R inhibitors. it may also be beneficial to the patients suffering with long term statin therapy and subsequent adverse effects.

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